Identification of host cellular requirements for positive strand RNA viruses: Hepatitis C virus and hepatitis E virus

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Dedicated to my

Grand mother Mary George

Abbreviations

The following list does not contain abbreviations that do not require definition according to the instructions for authors of the FEBS Journal available online at the web address http://www.blackwellpublishing.com/products/journals/suppmat/ ejb/ejbtab4.htm. Such abbreviations are used in the text and the legends of figures and tables without prior definition.

aa	Amino acid
AARE	Aminoacid responsive element
Ad	Adeno virus
AGMAT	Agmatine ureohydrolase
AH	Amphipathic helix
ALB	Albumin
AMV	Avian Myeloblastosis Virus
ARG1	Arginase, liver
ATF6	Activating transcription factor 6
Bcl2	B-cell lymphoma 2
BSA	Bovine serum albumin
CHOP	CCAAT/enhancer-binding protein homologous protein
CIAP	Calf intestine alkaline phosphatase
CsCl	Cesium Chloride
ChikV	Chikungunya virus
CMV	Cytomegalovirus
CPE	Cytopathic effect
ddH_2O	doubly deionized water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleoside triphosphate
DTT	Dithiothreitol
DYNC1H1	Dynein, cytoplasmic 1, heavy chain 1
ER	Endoplasmic reticulum
ERSE	Endoplasmic reticulum stress responsive elements
ECL	Enhanced chemiluminescence

FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
fwd	Forward
Fig	Figure
FRET	Fluorescence Resonance Energy Transfer
GFP	Green fluorescent protein
GRP	Glucose regulated protein
HAV	Hepatits A virus
HCV	Hepatits C virus
HBV	Hepatits B virus
HCVcc	Cell culture derived hepatitis C virus
HCVpp	Hepatits C virus pseudoparticles
HEV	Hepatitis E virus
HRP	Horseradish peroxidase
hpt	Hours post transduction
Hsp	Heat schock protein
IEM	Immuno electron microscopy
IP	Immuno precipitation
INPPL1	Inositol polyphosphate phosphatase-like 1
ITR	Inverted terminal repeats
KIFC1	kinesin family member C1
Kb	Kilo base
kDa	Kilo Dalton
LB	Luria-Bertani broth
LDLRAP1	low density lipoprotein receptor adaptor protein 1
MOI	Multiplicity of infection
MW	Membranous web
NBM	Nucleotode binding motif
NCBI	National Center for Biotechnology Information
NS4B	Non-structral protein 4B
OD	Optical density (absorbance/extinction)
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PI	Post infection
PIK	Phosphatidylinositol kinase-related kinase
PIP5K1A	phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
qRT	Quantitative real time
Rdrp	RNA-dependent RNA polymerase
Rev	Reverse
RIPA	Radioimmunoprecipitation assay
rpm	revolutions per minute
Rev	Reverse
RIG1	Retinoid-inducible gene 1
RT	1. reverse transcriptase
	2. reverse transcription
	3. room temperature
SREBPs	Sterol regulatory element-binding proteins
TBP	Tata binding protein
TG	Thapsigargin
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl)aminomethane
UTR	Untranslated region
UPR	Unfolded protein response
WB	Western Blot

Contents

1. Preface

Positive strand RNA viruses represent the largest group among the known genetic classes of viruses. These viruses are known to cause major epidemics among the human population. The major pathogens belonging to this group are Hepatitis C virus, severe acute respiratory syndrome (SARS) virus, West Nile virus, poliovirus, Dengue virus, Hepatitis E virus, Chikungunya virus and numerous plant and animal viruses. Most of these viruses share common features and require host factors for viral entry to virion assembly and release of the new virus. The outcome of the host pathogen interactions either is a potential target the host antiviral machinery or viral evasion mechanisms may act as a beneficiary for these viruses. So the identification of potential host candidates is relevant for elucidating the strategies of viral infection and also to develop new drug targets.

Positive strand RNA viruses comprise both the enveloped and non-enveloped viruses. The entry of enveloped virus like hepatitis C virus occurs mainly through the interaction between the viral envelope proteins and the cell surface receptors. These receptor interactions will trigger the activation of various host siganlling pathways and these may augment the successful entry of the virus into the cell. Here we used the genomics approach to identify the changes in the expression patterns of potential candidate genes during HCV entry. After succesful entry, the virus requires host factors to replicate themselves inside the host cellular environment. Replication of HCV occurs inside the membranous web mainly to evade host immune attacks. The non-structral protein (NS4B) of HCV is resposnsible for the formation of the membranous web through the rearrangement of cellular membranes. Since the host cellular targets of NS4B are largely unknown we have analyzed the activation and modultaion of cellular pathways by genomic and proteomic approaches.

The capsid protein of the non-enveloped hepatitis E virus known as ORF2 is currently used as a vaccine candidate. ORF2 protein is known to interact with cellular proteins and also modulates the regulation of several stress responsive genes. Here we have analyzed the mechanisms of the ER stress mediated pro-apoptotic effects caused by the HEV ORF2 and its potential role for the activation of anti-apoptotic activity of the host cell.

Chapter 1

2. Hepatitis C virus

2.1. Hepatitis C virus history and epidemiology

In 1970 diagnostic assays became available for the detection of hepatitis A and B viruses in 1970 (Coursaget *et al.*, 1979). Despite screening for hepatitis A and B viruses and it soon became clear that considerable number of most hepatitis cases of were not caused by infections with these or other known viral agents. These hepatitis cases not caused HAV or HBV were referred to as "non-A, non-B hepatitis" (NANBH) (Choo *et al.*, 1989). The first evidence of a virus associated with hepatitis was found in experiments with chimpanzees that showed a hepatitis after transfer of serum from patients with NANBH with identified by electron microscopy and named the hepatitis C virus (HCV) (Shimizu *et al.*, 1979). In 1989, Michael Houghton and colleagues isolated a cDNA clone of HCV, soon after the first Enzyme linked immunoadbsorbant based assay (ELISA) to detect the anti-HCV antibodies became available (Choo *et al.*, 1989; Kuo *et al.*, 1989). In the past 2 decades studies on HCV variability has led to the identification of 6 different major genotypes with more than 50 subtypes (Zein & Persing, 1996). HCV is a major health concern world-wide. The prevalence HCV infection is estimated to be 3% worldwide (Fig. 1). The major routes of HCV transmission occurs though the transfusion of infected blood and blood products. Other risk factors for the transmission include injection drug use (IDU) (Kaldor *et al.*, 1992; McCaughan *et al.*, 1992), tattoos (McCaughan *et al.*, 1992), needle stick accidents (Kiyosawa *et al.*, 1991; Mitsui *et al.*, 1992) or the transplantation of HCV infected organs (Pereira *et al.*, 1992). HCV transmission from mother to infant is possible at low rates (Martin & Denis, 1994). According to estimates of the World Health Organisation 130-170 million people or 2-3% of the worlds population are infected with HCV (2000; Shepard *et al.*, 2005). The epidemiological data on HCV infection show different rates of prevalence with respect to geographic areas. There are an estimated 400,00 chronically infected patients in Australia and Oceania,14 million in Americas, 16 million in Middle east,, 28 million in Africa, 17.5 million in Europe and 83 million in Asia (Martinson *et al.*, 1996). Prevalance studies based on the antibodies to HCV have shown that eastern Europe scores from1.5% to 2.5%, Western Pacific region from 2.5% to 4.9%, and those from the Middle east and Central Asia from 1% to more than 12% (1997). The epidemiology trends of the HCV infection in various countries have been studied. In china the geographical distribution of HCV infection is heterogeneous, with clear differences between the rural and urban area (Zhang *et al.*, 2010; Zhang *et al.*,

2005). Among the asian countries, Egypt has very high prevalence rates for HCV infection with 32% of the population being positive for HCV antibodies (el-Sayed *et al.*, 1996; el Gohary *et al.*, 1995).

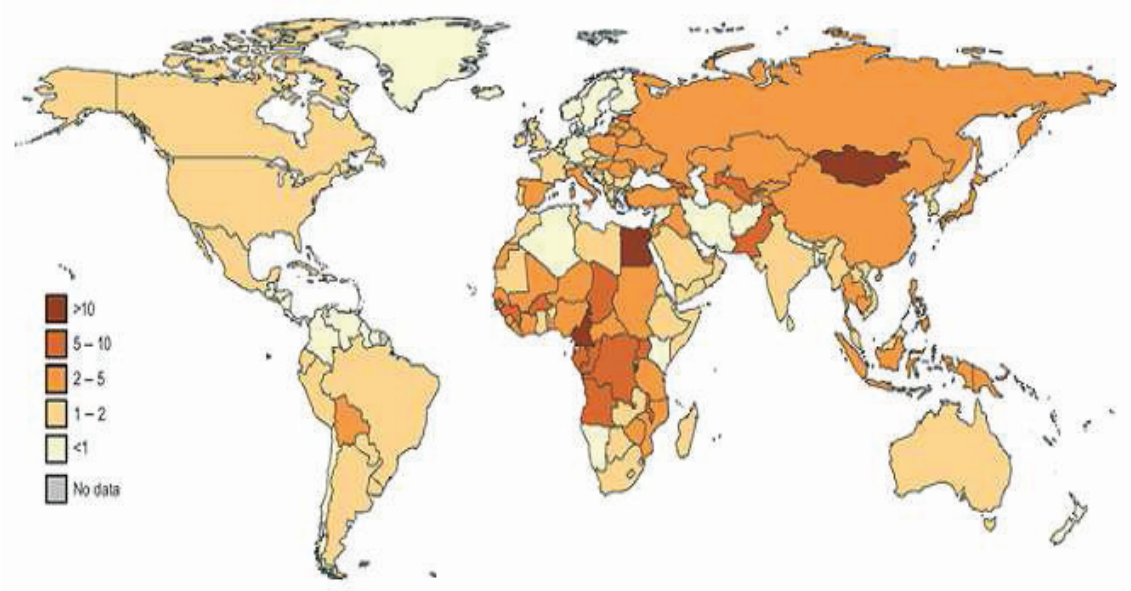

Figure 1: Global prevalence of HCV.(Lavanchy, 2010) (Adapted with permission from John Wiley and Sons publishing group, License Number: 2675281252670)

2.2.Genome organization of HCV

The genome of HCV is a single stranded RNA molecule with positive polarity. The genome codes for only one open reading frame (ORF) which encodes a polyprotein of 3000 aminoacids which is flanked by two UTR at the 5' and 3' end. The length of the 5' UTR is about 341 nucleotides (nts) and has an internal ribosome entry site (IRES) which initiates the polyprotein translation in a cap independent manner (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1995). The 3'-UTR of the positive-strand contains a short poly (U/UC) tract, and a highly conserved 98 nucleotide RNA element essential for replication (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003). Recent studies also found that cis acting replication element (CRE) consists of a stem loop designated as SL3.2 within a larger cruciform RNA element designated as SL3 RNA (Friebe *et al.*, 2005; You *et al.*, 2004). The only ORF of HCV consists of approximately 9000 nucleotides. Post translational processing by cellular and viral proteases yields 11 proteins including the core or viral nucleocapsid and the envelope proteins (E1 and E2) (Grakoui *et al.*, 1993). The release of structural proteins from the polyprotein precursor is mainly through the endoplasmic reticulum (ER) signal peptidase. HCV core protein is localized in the cytoplasm and is bound to the ER memebranes but a small proportion of the core protein will also be

present in the nucleus. The envelope proteins E1 and E2 are glycosylated and form a noncovalently linked complex (Ralston *et al.*, 1993). The envelope proteins are necessary for the viral attachment, entry and fusion events of HCV (Lavie *et al.*, 2007). The separation of the structural proteins from the non structural proteins by a 63 aminoacid polypeptide (P7), is cleaved from E2. The two transmembrane domains of P7 is connected by a short hydrophilic segment. P7 can form hexamers and is known to have ion channel activity(Pavlovic *et al.*, 2003). Recent studies have reported an additional HCV protein designated as F protein (F) or alternative frame protein (ARFP) of up to 160 aminoacids (Varaklioti *et al.*, 2002; Walewski *et al.*, 2001). However, the function and role of this protein still has to be evaluated. The non structural proteins 2 (NS2) to non structural protein 5B (NS5B) are involved in polyprotein processing and viral replication. The NS2/NS3 junction is cleaved by the NS2/NS3 autoprotease, azinc dependent metallo-proteinase and the NS3 serine protease located in the N-terminal region of NS3 (Lorenz *et al.*, 2006; Pallaoro *et al.*, 2001). In addition an NTPase/RNA helicase domain is also found in the C-terminal two thirds of NS3. NS4A functions as a cofactor for the NS3 serine protease and is incorporated into the enzyme core (Lin *et al.*, 1994b; Tanji *et al.*, 1995). NS4B is an integral memebrane protein which is known to cause specific cellular membrane alterations for the establishment of membranous web formation which later serves as the site for HCV replication (Egger *et al.*, 2002; Gosert *et al.*, 2003; Moradpour *et al.*, 2004). NS5A is a membrane associated phopshoprotein of 56 kDa and 58 kDa in the hyperphosphorylated form (Kaneko *et al.*, 1994). (Kaneko *et al.*, 1994). The role of NS5A in the viral life cycle still has to be elucidated. HCV replication proceeds via the synthesis of complimentary minus strand RNA using the genome as a template and the subsequent synthesis of genomic plus strand from this minus strand. These steps will be catalyzed by the NS5B encoded RdrP (Ivashkina *et al.*, 2002). Furthermore cis-acting RNA elements in the coding region of NS5B, which form a kissing loop and their nteraction with SL II in the X-region are essential for HCV RNA replication (You *et al.*, 2004).

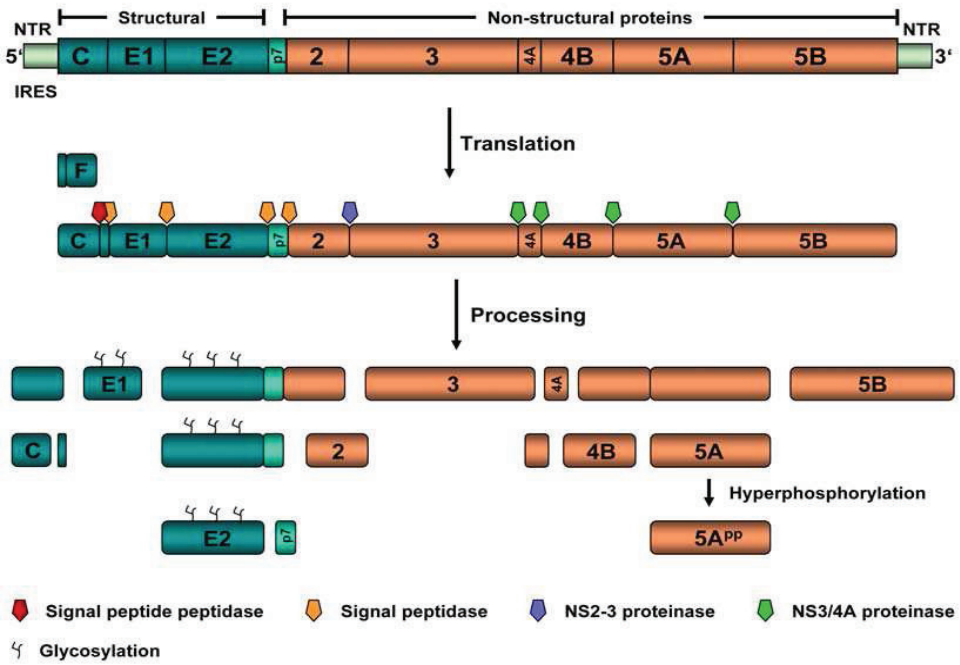

Figure 2: Organization of the HCV genome and processing of the HCV polyprotein. The HCV genome with the 5'- and 3'-UTRs is shown in the top. Translation products of the HCV genome are drawn below. The processing of the polyprotein is shown at the bottom of the picture. The cleavage of major pathways leading to distinct processing intermediates, most notably E2-p7-NS2 and NS4B-5A are indicated. The proteolytic cleavage is probably due to the hyperphosphorylation of NS5A. The branched lines represent the glycosylation of E1 and E2. Ribosomal frameshifting will lead to the generation of the F protein that is depicted above the polyprotein. (Bartenschlager et al., 2004) (Adapted with permission from Elsevier publishing group, license Number: 2661970744585).

2.3. Experimental systems to study HCV

Historically, one of the major difficulties in HCV research and drug development has been a lack of experimental systems to study the life cycle of HCV *in vitro*. However, different model systems have been developed now and are successfully used to study the different aspects of HCV life cycle. The most important steps in the development of HCV in vitro systems mainly included the development of replicons, HCV pseudotyped particles, and the recently developed infectious cell culture system. In 1999 Lohman *et.al*., developed the genotype 1b HCV replicon system (Lohmann *et al.*, 1999). Later the sub genomic and full length replicons were fused with exogenous reporters like luciferase, alkaline phopshatase, beta lactamase that enabled the development of reporter based HCV replication screnning assays (Yi *et al.*, 2002; Yi & Lemon, 2002) (Krieger *et al.*, 2001). Even though the replicons are still in use, the study of viral entry

steps was not possible with this system until the development of HCV pseudoparticles (HCVpp). HCVpp which incorporate HCV E1 and E2 glycoproteins on to the retroviral cores of murine leukemia virus (MLV) or Human immuno deficiency virus (HIV) (Bartosch *et al.*, 2003a; Hsu *et al.*, 2003) has the serological properties and mimics the entry mechanism of HCV. This system was a breakthrough in HCV research to identify cellular factors and receptors necessary for the viral entry process. In addition the HCVpp system helped to identify neutralizing epitopes for HCV and has been used to screen for entry inhibitors of HCV.

A cellculture system which allowed the study of the whole viral life cycle became available after the cloning of a genotype 2a genome from a patient with fulminant hepatitis in 2005. Several groups reported the production of a infectious cell culture produced HCV which was based on the JFH-1 virus genotype 2a (Wakita *et al.*, 2005; Zhong *et al.*, 2005). The viral particles were generated after transfection of Huh-7 cells with full-length RNA from the JFH-1 genome. This tool has greatly helped to study the various events of the HCV life cycle like viral entry, viral assembly and release. In addition, this system is used extensively for the screeningof various inhibitors of all stages of the viral lifecycle.

2.4. Life cycle of HCV

HCV infection begins with the entry process which involves the HCV structural proteins on the viral side and the receptor molecules at the surface of target cells. The two envelope glycoproteins, E1 and E2, are the major components of the HCV envelope which is responsible for the viral entry and fusion events (Hsu *et al.*, 2003). E1 and E2 are type I transmembrane glycoproteins, they form a heterocomplex and bind to the cell surface receptors. Several receptors have been proposed to mediate HCV binding or HCV binding and internalization. Among the known receptors for HCV infection, four of them were well studied like cluster of differentiation 81 (CD81), scavenger receptor class B (SR-B1), Claudin 1, and a low-density lipoprotein receptor (LDL-R) (Agnello *et al.*, 1999; Bartosch *et al.*, 2003b; Evans *et al.*, 2007; Pileri *et al.*, 1998). CD81 and SR-B1 are known to lead entry by specific interaction with E1 or E2 (Wunschmann *et al.*, 2000). The large extracellular loop of CD81 has been shown to bind to the envelope glycoprotein E2 of HCV. Claudin-1 appears to act late in the entry process, after the interaction with CD81 (Evans *et al.*, 2007). After binding to the specific receptors, the HCV nucleocapsid is released into the cell cytoplasm through a series of fusion events between viral and cellular membranes (Fig 3). HCV entry into cells has been shown to

be pH dependent mainly through clathrin-mediated endocytosis. Entry of HCV is followed by a fusion step within an acidic endosomal compartment and followed by the release of the icosahedral nucleocapsid into the cytoplasm (Tscherne *et al.*, 2006). The decapsidation of viral nucleocapsid releases the positive-sense HCV singlestranded (ss) RNA genome into the cytoplasm. The genome is recognized and translation starts by a cap independent internal translation by exploiting the host machineries like eukaryotic elongation initiation factors (elF) 2, 3 and other viral proteins (Friebe *et al.*, 2001; Luo *et al.*, 2003).

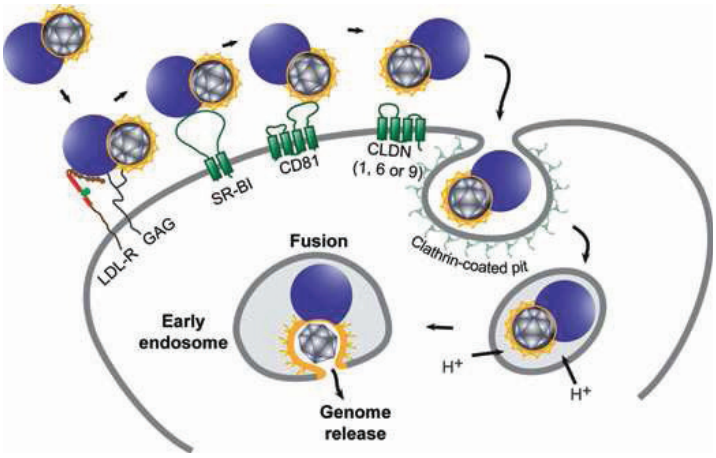

Figure 3: Model of HCV entry into hepatocytes. (Dubuisson *et al.*, 2008)**.** (Adapted with permission from John Wiley and Sons publishing group, License Number: 2670680696561)

The cleavage of the ORF by host and viral proteases leads to ten functional viral proteins. The structural proteins are liberated by a host signal peptidase (SP); which cleaves the C/E1 and E1/E2 junctions (Carrere-Kremer *et al.*, 2004; Lin *et al.*, 1994a). The cysteine protease dimer NS2 that cleaves at the NS2/NS3 junction and the other cleavage sites are processed by NS3 but requires activation by NS4A for the cleavage of the NS4B/NS5A junction (Lorenz *et al.*, 2006). After the polyprotein processing, the NS proteins of HCV lead to the formation of replication complexes or membranous webs. This process requires the cellular rearrangements of the host's milieu interne. NS4B is known to be the key regulator for the formation of the membranous web, derived from the (ER) membranes. The membranous web contains small vesicles embedded in a membranous matrix, forming a membrane-associated multiprotein complex that contains all of the non-structural HCV proteins (Egger *et al.*, 2002; Kasprzak *et al.*, 2005; Lundin *et al.*, 2006). After translation, NS5A an RNA-binding protein, is hypothesized to bind to the template RNA providing a link to replication (Huang *et al.*, 2005). The RNA-dependent RNA polymerase encoded by NS5B, synthesizes a intermediate negative

strand and then transcribes new genomes to be used for translation and virus production (Behrens *et al.*, 1996). The NS3 helicase and host cell helicases unwind secondary structures in single strand ribo-nucleic acid (ssRNA) and uncouple template-transcript double-stranded (ds) RNA (Goh *et al.*, 2004; Isken *et al.*, 2007). NS4B can induce structural rearrangements in the ER membrane leading to formation of a 'membranous web' structure (Egger *et al.*, 2002; Lundin *et al.*, 2006; Mottola *et al.*, 2002). The N-terminal transmembrane domain of NS4B mainly mediates the rearrangement process of the membrane and the proper insertion of this domain relies upon NS3-mediated proteolysis (Lundin *et al.*, 2006). This rearrangement process may help the virus to escape from the host anti viral defence strategies (Hiscott *et al.*, 2006; Li *et al.*, 2005). Assembly of the virus can occur on lipid rafts. The assocatiaon of the C protein with lipid droplets requires the host intramembrane protease signal peptide peptidase (Ma *et al.*, 2007). The C protein usually associated with positive-sense RNA genome assembles to nucleocapsids. The nucleocapsid will be enveloped with a double membrane and glycoproteins, E1 and E2 from the ER. Finally the enveloped viruses are released from the host cells mainly through the exocytotic pathways. p7 is a small protein, forms an ion channel in the ER and is essential for viral assembly and release (Griffin *et al.*, 2003; Murray *et al.*, 2007; Steinmann *et al.*, 2007). Recent studies have shown a role of NS2 for viral assembly and release, independent of its protease function (Jones *et al.*, 2007). Other non-structural protein like NS5A also have been reported to interact with C and other host proteins in a phosphorylation dependent manner to mediate the viral assembly process of HCV (Appel *et al.*, 2008; Tellinghuisen *et al.*, 2008).

2.5. Known host factors during HCV entry

All viruses include HCV are known to exploit the host cell machinery for their survival in each phase of the viral life cycle. Compared to viruses, host cells have more genes and more functionally diversified classes of genes. Thus, most of the steps during the HCV infection involve subsequential interactions between viral components and host cell components. Accordingly, host factors play an important role in most steps of viral infection and identification of such bona fide cellular targets and their contribution to viral entry has long been considered as an important frontier. Identification of relevant receptors for HCV has led to further investigations into the entry mechanisms of HCV. According to the post binding mechanisms of entry, the enveloped viruses may use either endocytotic pathways or enter the cell via fusion of the viral envelope with the cellular plasma membrane. The acidic pH of the

endosomes can trigger viral fusion events during endocytosis and studies using HCV pseudoparticles has shown that HCV entry is pH dependent (Bartosch *et al.*, 2003b; Hsu *et al.*, 2003). The functional role of clathrin mediated endocytosis during HCV entry was assessed by siRNA mediated silencing of clathrin heavy chain and using clotrimazole an inhibitor of clathrin coat pit formation. These studies confirmed that HCV uses clathrin mediated endocytosis for the entry process into host cells. Also the silencing of claudin 1 inhibited the replication of HCV in hepatoma cells. These observations indicated that tetraspanin CD81 is required but not sufficient for entry and claudin1 is a critical cofactor for the entry process of HCV (Meertens *et al.*, 2008).

Many viruses including HCV will exploit the intracellular membrane trafficking pathways during each phase of their life cycle. Delivery of the RNA genome into the host cell requires successful entry and intracellular trafficking in a hepatocyte. The identification of host factors for HCV entry will lead to the discovery of future drug targets to inhibit the entry of HCV. siRNA based screening methodology has been widely used by HCV researchers to investigate the role of genes involved in cellular membrane trafficking during HCVentry (Lupberger *et al.*, 2011). These studies have clearly shown that HCV infection was partly blocked upon the knockdown of the gene sets like phosphatidylinositol 4-kinase type III alpha (PI4KIIIα), EPSIN1, Adaptor related protein 2 1 subunit (AP21). Among these genes, EPSIN1 and adaptor protein are involved in the clathrin mediated ednocytosis. The involvement of PI4KIIIα in HCV entry and replication has also been reported by several groups (Berger *et al.*, 2009; Borawski *et al.*, 2009; Trotard *et al.*, 2009). The role of actin in HCV entry has been investigated by several groups. Fluorescence microscopy revealed that virus particles were associated with the filopodia at early timepoints protruded from the cell surface. Recenlty Glen Rendall and his team developed the single particle trafficking analysis for HCV endocytosis and found the sequential interactions of HCV virions with the actin cytoskeleton, during the migration of HCV virions inside the cell after entry. This group also found the co localization of HCV with clathrin and the ubiquitin ligase c-Cbl before the internalization events. After successful internalization, the HCV particles were transported with the Rab5a containing endosomes, which also shows that HCV uses the early endosomes for intracellular trafficking.(Coller *et al.*, 2009). The cortical actin cytoskeleton showed no specific morphological changes in compared to the uninfected cells (Brazzoli *et al.*, 2008). Thus, the indentification of new host factors for HCV infection will pave the way for the development of novel antiviral therapies.

2.6. NS4B

2.6.1. General features of NS4B

HCV NS4B is a 27 kDa protein whose role in viral life cycle has been been characterized completely. Based on *in silico* prediction algorithms and experimental data the NS4B protein of all flavivirus family members belongs to the category of integral membrane proteins with many transmembrane segments (Gouttenoire *et al.*, 2010). NS4B protein is liberated from the polyprotein durng the processing events by the serine protease activity of NS3-4A. These clevage events which occur at the NS4A/NS4B and NS4B/NS5A junction were reported as the last polyprotein processing events (Bartenschlager *et al.*, 1994). Localization studies using a NS4B-GFP fusion protein has shown that NS4B is oriented towards the cytosolic side of the ER and it seems to form cytoplasmic dot like structures or foci (Elazar *et al.*, 2004; Gretton *et al.*, 2005; Hugle *et al.*, 2001; Lundin *et al.*, 2003). The ultrastructral investigations revealed that these dot like structures correspond to memebrane vesicles embedded in a matrix which was later designated as molecular web (MW) (Egger *et al.*, 2002; Gosert *et al.*, 2003). The MW is the major site for HCV RNA synthesis in the host cells (Gosert *et al.*, 2003). Electron microscopy examination has shown that the sponge like inclusions observed in the MW of HCV infected liver of chimpanze were associated with the viral non structural proteins (Pfeifer *et al.*, 1980). Several groups have also reported the presence of an amphipathic helix 1 (AH1) and amphipathic helix 2 (AH2) (Elazar *et al.*, 2004), which is known to clench the membrane association. This interaction is required for viral replication.The AH has also been reported to have the potential to traverse the phospholipid bilayer as a transmembrane domain (Cho *et al.*, 2010; Gouttenoire *et al.*, 2009a). NS4B consists of four trasnmebrane segments (Hugle *et al.*, 2001; Lundin *et al.*, 2003; Welsch *et al.*, 2007), an N-terminal part (aminoacids 1 to 69), central part four transmebrane passages (aminoacids 70 to 190) and a C-terminal part (aminoacids 191 to 261). 3D structre analysis of AH2 have shown the twisted amphipathic helix extending from amino acids 229 to 253 (Gouttenoire *et al.*, 2009b) and this mediates the membrane association. Therefore, membrane association of HCV NS4B is also mediated by the N and C-terminal parts of the protein (Guillen *et al.*, 2010; Liefhebber *et al.*, 2009). It is also reported that the palmitoylation sites at the C-terminus does not have effect on the replication of HCV (Yu *et al.*, 2006). Along with other nonstructural HCV proteins, NS4B is also known to form oligomers. The evidence for inter and intramolecular contacts involved in

the oligomerisation of NS4B was provided by fluorescence resonance energy transfer (FRET) and co-immunoprecipitation methods (Yu *et al.*, 2006).

2.6.2. Role of NS4B in HCV replication

The best documented and established function of NS4B is the induction of the MW through intracellular rearrangements. The MW is the primary hub for the HCV replication. However, the molecular mechanisms leading to the formation the MW following NS4B expression is still unknown. Recent studies suggest that the amphipathic helix 2 within the N terminus of NS4B plays an important role in the formation process leading to the MW. Interaction of NS4B AH2 with lipid vesicles composed of 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) clearly shows that NS4B AH2 mediates both helix oligomerization and specific aggregation of lipid vesicles. The structures formed due to the vesicle aggregating activity are reminiscent of the MW (Gouttenoire *et al.*, 2009b). The vesicle aggregating activity of NS4B AH2 was monitored by automated fluorescent microscopy and quantified the amount of fluorescent signal contained within the NS4B AH2 lipid vesicle aggregates (Cho *et al.*, ; 2010). This relatively simple in vitro system for NS4B activity has been used for the screening and identification of several candidate pharmacological inhibitors of lipid vesicle aggregation. Additional results showing the importance of NS4B were shown in the replicon system. Site directed mutagenesis of of Lys 135 to Thr of NS4B from the genotype 1b Con1 strain was shown to significantly enhance HCV RNA replication in Huh-7cells (Lohmann *et al.*, 2003). Further more, it has been shown that NS4B can transcomplement the RNA replication and also it contributes to the processes engaged in virus assembly and release (Jones *et al.*, 2009). In vitro studies have demonstrated the interaction of HCV RNA with NS4B at the 3' end of the viral genome negative strand RNA. In addition, the NS4B protein of HCV has been shown to contain a nucleotide binding motif (NBM) located between transmembrane domains located between 2 and 3, (Einav *et al.*, 2004; Thompson *et al.*, 2009). The nucleotide binding motiff of HCV NS4B has been shown to hydrolyse GTP and ATP, which implicates that NS4B also shows an adenylate kinase activity (Thompson *et al.*, 2009). The nucleotide binding motiff of NS4B is well conserved among various genotypes and isolates. Mutational analysis of this nucleotide binding motif motiff also inhibited the the HCV replication (Einav *et al.*, 2004).

2.6.3. Interactions of NS4B with the host cell

HCV NS4B has been shown to interact with the host cellular machinery for the productive needs of the HCV life cycle. NS4B will play a major role because of its multispanning membrane topology and its capacity to alter host cellular membranes. Expression of NS4B protein causes ER stress and activation of unfolded protein response (Li *et al.*, 2009; Zheng *et al.*, 2005). It is well established that activation of UPR will lead to the activation of three signaling cascades which involves the RNA-activated protein kinase-like ER kinase, inositol-requiring kinase 1 and the basic leucine zipper-containing activating transcription factor 6 (ATF6). Of the proteins executing these signaling cascades, NS4B is known to interact directly with both ATF6 alpha and beta (Tong *et al.*, 2002). It is well known that HCV infection is often associated with hepatic steatosis but the molecular mechanisms are not fully understood (Hwang *et al.*, 2001). The depletion of cholesterol levels in cells will usually activate the proteolytic cleavage of sterol regulatory element binding proteins (SREBPs) which in turn activates the genes related to cholesterol and fatty acid metabolism (Sato, 2009). Recent evidences shows that NS4B can induce the SREBP cleavage pathway and further activation of fatty acid synthase upregulation and lipid accumulation is dependent on the phosphoinositol-kinase pathway (PIK) (Park *et al.*, 2009b). Hence, the NS4B induced modulation of lipid metabolism reflects the necessity of the HCV life cycle on lipid constitutents for membrane rearrangements. Overexpression of NS4B in NIH3T3 cells was found to transform the cells in cooperation with the oncogene H-Ras (Park *et al.*, 2000). More recently it has been shown that that the nucleotide binding motif of NS4B can mediate the transformation and tumour formation without the involvement of H-Ras oncogene (Einav *et al.*, 2008). However, transgenic mice expressing NS4B did not show any tumour development. Further studies are needed to explore the contribution of NS4B to the mechanisms of hepatic carcinogenesis. Some family members of the *flaviviridae* including West nile,Yellow fever and Dengue viruses were shown to repress antiviral host defenses by inhibiting type I interferon signaling (Munoz-Jordan *et al.*, 2005; Munoz-Jordan *et al.*, 2003). Interestingly, NS4B is known to inhibit the retinol induced gene 1 (RIG1) mediated interferon activation and in addtition NS4B can also inhibit the antiviral activity of interferon alpha (Tasaka *et al.*, 2007; Xu *et al.*, 2009). These findings suggest a role for NS4B in the evasion from the innate immune response. More work is needed to understand the complex interaction between NS4B and the host cell.

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2.7. Materials and Methods

2.7.1. Devices

Camera	Olympus, Hamburg
Cell incubator	Type B 5060 EC CO_2, Heraeus, Hanau
	Labotect, Göttingen
Centrifuge	Eppendorf, Hamburg
	Biofuge fresco, Heraeus
	AllegraTM 21R, Beckman Coulter, Fullerton USA
Electroblotting chamber	Bio-Rad, München
Electrophoresis chamber	For agarose gels: Wide mini sub cell, Bio-Rad, München
	For polyacrylamide gels: mini protean 3 Bio-Rad, München
Heating block	DRI-BLOCK DB3, Techne, Jahnsdorf
Incubator	Heraeus, Hanau, Arbeitsbank, BDK Luft- und Reinraumtechnik Sonnenbuhl-Genkingen
Laminar Flow	Arbeitsbank Heraeus-Kendro, Hanau
Minishaker	MS2, IKA, Staufen
Microscopes	Axiovert 25, Carl Zeiss, Göttingen
	Axiovert 40 Carl Zeiss, Göttingen
Microwave oven	Micro-Chef FM 3915 Q, Moulinex
PCR Thermocycler	Personal Cycler, Biometra, Göttingen
	iCycler, Bio-Rad, München
pH-meter	pH 211, Microprocessor pH meter, Hanna-instruments, USA
Pipettors	Finn pipettors (5-40 µl, 40-200 µl, 200-1000 µl), Labsystems, Finnland
	Eppendorf pipettors (0.5-10 µl, 10-100 µl, 200-1000 µl), Eppendorf, Hamburg

Power supply	Power Supply Model 3000 X, Bio Rad, München
Shaker	Vortex Genie, Bender & Hobein, München
Sonicator	Bandelin Electronic, Berlin
Spectrophotometers	Smart SpecTM3000, Bio-Rad, München Spectra, Tecan, Crailsheim
Table top centrifuge	Centrifuge 5415 C, Eppendorf, Hamburg
UV transilluminator	Bio-View, Biostep, Jahnsdorf
Water bath	GFL1086, GFL, Wunstorf

2.7.2. General material

Cryo tubes, 2 ml	Greiner, Frickenhausen
Culture dishes, 35 mm diameter	Becton Dickinson, Heidelberg
Culture dishes, 90 mm diameter	Nunc, Wiesbaden
Culture flasks, 80 cm^2	Nunc, Wiesbaden
Filter paper (Whatman 3 MM)	Whatman, Göttingen
Filtration units (sterile Millex units)	Millipore, Eschborn
Glass pipettes, 1 ml, 5 ml, 10 ml	Hirschmann, Eberstadt
Glass ware	Schott, Mainz ; Brand, Wertheim
Syringes, 20 ml, sterile	Braun, Melsungen
Nitrocellulose membrane	Bio-Rad, München Amersham, Freiburg
PCR tubes (0.2 ml)	PeqLab, Erlangen
Petri dishes (AD94/H16 mm)	Roth, Karlsruhe
Pipette tips	Braun, Melsungen
Plastic tubes, 14 ml	Greiner, Frickenhausen
Plastic tubes, 50 ml	Nunc, Wiesbaden
Plastic reaction tubes, 1.5 ml	Brand, Wertheim
Pursept®-A disinfectant solution	Merz via Fischer, Frankfurt a. m
Safe Skin Satin Plus powder-free latex gloves	Kimberly Clark, Koblenz-Rheinhafen
Sterile filters (0.2 µm and 0.45 µm)	Renner GmBH, Dannstadt
Affymetrix HGU133 PLUS 2.0 Gene Chip	Affymetrix, USA
Sterile single use serological pipets (10 ml)	Falcon via Multimed, Kirchheim Teck

X-ray film	Amersham, Freiburg

2.7.3. Chemicals

Ammonium peroxodisulfate (APS)	Fluka, Steinheim
Bradford assay dye reagent	Bio-Rad, München
Bromophenol blue	Fluka, Steinheim
BSA	Sigma-Aldrich, Steinheim
Calcium chloride dehydrate	E. Merck, Darmstadt
Coomassie brilliant blue R 250	Sigma-Aldrich, Steinheim
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Steinheim
EDTA	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Mercaptoethanol	Sigma-Aldrich, Steinheim
$MnCl_2$	Sigma-Aldrich, Steinheim
NaCl	Sigma-Aldrich, Steinheim
$NaHCO_3$	Sigma-Aldrich, Steinheim
PageRuler™ prestained protein ladder	MBI Fermentas
Potassium chloride	E. Merck, Darmstadt
SDS	Fluka, Steinheim
Sucrose	Roth, Karlsruhe
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Steinheim
Protease inhibitor cocktail EDTA free	Thermo Scientific, USA
RIPA buffer	Thermo Scientific, USA
Tris	Roth, Karlsruhe
Tween® 20	Fluka, Steinheim
Thapsigargin	Tocris, USA

2.7.4. Kits

Enhanced chemiluminescence (ECL) detection reagent	Amersham, Freiburg
high pure viral RNA kit	Roche, Berlin

QIAquick gel extraction kit	Qiagen, Hilden
QIAquick PCR purification kit	Qiagen, Hilden
RNeasy RNA isolation kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
MEGAscript® T7 Kit	Ambion, Applied biosystems, California, USA
m^7G Capping System	Epicentre Biotechnologies, USA

2.7.5. Reagents for molecular biology

Agarose	PeqLab, Erlangen
BSA (100x)	New England Biolabs, Frankfurt a.M.
Dithiothreitol (DTT), 0.1 M	Gibco BRL, Karlsruhe
GeneRuler 1 kb DNA ladder	MBI Fermentas, St. Leon-Rot
GeneRuler 100 bp DNA ladder plus	MBI Fermentas, St. Leon-Rot
Deoxyribonucleoside triphosphate (dNTP; 10 mM each)	PeqLab, Erlangen
Ethidium bromide	Sigma-Aldrich, Steinheim
PCR primers	TIB MOLBIOL GmbH, Berlin
	Eurofins MWG Operon, Ebersberg
RNAsin ribonuclease inhibitor (40 U/µl)	Promega, Mannheim
T4 DNA Ligation Buffer (10x)	MBI Fermentas, St. Leon-Rot
	New England Biolabs, Frankfurt a.M.
Turbofect transfection reagent	Fermentas GmbH
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen, Karlsruhe

2.7.6. Enzymes for molecular biology

Restriction enzymes	New England Biolabs, Frankfurt a.M.
Calf intestine alkaline phosphatase (1U/µl)	New England Biolabs, Frankfurt a.M.
HotStart Taq DNA polymerase	Qiagen, Hilden
MLV Reverse Transcriptase	Qiagen, Hilden
Pfu turbo High-Fidelity DNA polymerase	Stratagene, Amsterdam
RNAse A	Qiagen, Hilden

T4 DNA ligase	MBI Fermentas, St. Leon-Rot New England Biolabs, Frankfurt a.M.

2.7.7. Constituents and reagents for bacterial and mammalian cell cultures

Antimycotic/antibiotic	Sigma-Aldrich, Steinheim
Carbenicillin, disodium salt	Roth, Karlsruhe
Dulbecco's modified Eagle's medium (DMEM) powder lacking pyruvate and $NaHCO_3$	GibcoBRL, Karlsruhe
Fetal calf serum (FCS)	Biochrome, Berlin
Kanamycin	Applichem, Darmstadt
LB-agar powder	Fluka, Steinheim
LB-broth powder	Fluka, Steinheim
Penicillin G, potassium salt	Serva, Heidelberg
Superoptimal broth, catabolite repression (SOC) medium	Novagen, Schwalbach/Ts.
Streptomycin sulfate	Serva, Heidelberg
Trypsin	ICN, Eschwege

2.7.8. Antibodies

2.7.8.1. Primary antibodies

His Probe, (rabbit) polyclonal antibody	Santacruz Biotechnology, USA
Beta catenin (rabbit) polyclonal antibody	Cell Signaling Technology, Danvers, USA
Phospho-Beta catenin (rabbit) polyclonal	Cell Signaling Technology, Danvers, USA
Beta actin (Mouse) monoclonal antibody	Sigma-Aldrich, Steinheim
TBP (Mouse) monoclonal antibody	Abcam

2.7.8.2. Secondary antibodies

Anti-Mouse IgG (Goat) HRP conjugated	Santacruz Biotechnology, USA

Anti-Rabbit IgG (Donkey) HRP conjugated	Santacruz Biotechnology, USA

2.7.9. Bacterial strains

Top10 *E.coli* cells	Invitrogen, Karlsruhe
BJ5183 *E.coli* cells	Stratagene, Amsterdam
NovaBlue Singles^{TM} competent cells	Novagen, Schwalbach/Ts.
Bl21 cells	Invitrogen, Karlsruhe

2.7.10. Mammalian cell lines

293 Human embryonic kidney cells	American Type Culture Collection (ATCC)
HepG2 cells	American Type Culture Collection (ATCC),
Huh7 cells	Provided by Ralf Bartenschlager
Hep3B	American Type Culture Collection (ATCC)

2.7.10.1. Vectors

pcDNA 3.1/v5-His-TOPO	Invitrogen, Karlsruhe
pET32a-His Trx	Kind gift of Ralf Bartenschlager, Heidelberg, Germany
pAdTrack-CMV	Addgene, Cambridge, USA

2.7.11. Cell culture

2.7.11.1. Cultures of the human hepatocellular carcinoma Huh-7

Starting of the culture

The Huh7 cell line was kindly provided by Prof Ralf Bartenschlager as a cryo stock. To take the cells into culture, the cryo stock was rapidly thawed at 37°C in a pre-warmed circulating water bath. Afterwards, the cell suspension was transferred to an 80 cm^2 culture flask containing 20 ml DMEM /10% FCS /5% Non Essential Aminoacids, supplemented with antimycotic solution and subsequently incubated at 37°C in an atmosphere of 95% air and 5%

CO_2 for 4 h. After attachment of the cells to the culture dish (approximately 4 hours after seeding), the medium was renewed by fresh DMEM / FCS supplemented with antimycotic and antibiotic.

2.7.11.2. Cultures of the the human embryonic kidney cell line HEK293

Starting of the culture

The HEK293 cell line was kindly provided by Anja Stoll as a cryo stock at passage number 5. To take the cells into culture, the cryo stock was rapidly thawed at 37°C in a pre-warmed circulating water bath. Afterwards, the cell suspension was transferred to an 80 cm^2 culture flask containing 20 ml DMEM /10% FCS supplemented with antimycotic solution and subsequently incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 4 h. After attachment of the cells to the culture dish (approximately 4 hours after seeding), the medium was renewed by fresh DMEM / FCS supplemented with antimycotic and antibiotic.

2.7.11.3. Generation of cell culture infectious HCV

The methods mentioned from 2.7.11.3 to 2.7.11.5 were completely done at the MRC, virology lab, Glasgow.

The JFH1 RNA was synthesized *in vitro* using the plasmid pJFH1 which was obtained from T.Wakita. Briefly, after trypsinization of the subconfluent Huh7 cells, the cells were harvested by centrifugation, washed twice, and resuspended in ice-cold PBS at 10^7cells/ml. *In vitro* synthesized JFH1 RNA (10 µg) was mixed with 0.4 ml of cells in a 0.4 cm gap Gene Pulser cuvette (Bio-Rad) and immediately pulsed once at 960-F and 270 V using a BioRad Gene pulser cell electroporator. After electroporation, cells were allowed to recover for 10 min at room temperature prior to the addition of complete medium and were then plated in a 10 cm diameter tissue culture dish. Filtered supernatants were either stored directly at (−70°C) or concentrated by ultracentrifugation on a sucrose cushion.

2.7.11.4. Purification of HCVcc by sucrose density-gradient ultracentrifugation

This filtered supernatant from 2.7.11.3 was stored at (−70°C). Alternatively, Virus particles released from transfected cells were pelletted by ultracentrifugation of the filtered supernatant through a 20% sucrose cushion at $1.0 \times 10^5 \times g$ for 3h at 4 °C. Pellets were resuspended in phosphate-buffered saline (PBS) and the resulting pelletwas resuspended in PBS and stored at (−70°C).

2.7.11.5. Synchronized infection using magnetic adsorption

The purified viruses were pre-incubated in PBS with either FluidMAG-DP for 2 min or CombiMAG for 20min, at 370g/ml and 37g/ml, respectively. Virus–MNP complexeswere then added to the target cells in medium. The cell dish was transferred onto appropriate magnets and kept under magnetic field for 2 min at room temperature. The cells were washed twice with medium and incubated further for 48 h or 72 h. This mixture was then added to the cells after removing all culture medium.

2.7.11.6. Total RNA isolation from Huh-7 cells

Total cellular RNA was isolated using RNeasy RNA isolation kit from Qiagen. The cells (approximately 10^7 cells) were collected by trypsinisation and pelleted by brief centrifugation. The cell pellet was disrupted by adding 600 µl of buffer RLT and repeated pipetting. Then 1 volume of 70% ethanol was added to the lysate, and the solution was mixed well by pipetting. The sample was transferred to an RNeasy spin column supplied with the collection tube and centrifuged (13,000 rpm, RT, 15 s). The flow-through was discarded and the membrane of the column was washed by adding 700 µl of buffer RW1 and a brief centrifugation (13,000 rpm, RT, 15 s). The flow-through was discarded and the membrane of the column again washed with buffer RPE (with ethanol) by centrifugation (13,000 rpm, RT, 1 min). After this step, the column was again briefly centrifuged (13,000 rpm, RT, 1 min) to prevent ethanol carry-over and to dry the column. The column was subsequently placed in a 1.5 ml sterile microfuge tube, and the total RNA was eluted with 50 µl of RNase-free water by centrifugation (13,000 rpm, RT, 1 min). The concentration of isolated RNA was measured photometrically at 260 nm.

2.7.12. Microarray

The host gene expression profile changes were analyzed after synchronized infection of HCVcc-CombiMAG, under magnetic field. As a control Huh7 cells were mock infected with CombiMAG alone. Total RNA samples from the infected Huh7 cells were hybridized to Human genome U133 plus 2.0 array (Affymetrix) interrogating 47,000 transcripts with more than 54,000 probesets. Huh7 cells were infected with HCVcc and the RNA was isolated at various time points like 0,15,30,60 and 180 minutes and the RNA samples were subjected for quality test. Array hybridization was performed according to the supplier's instructions using the Genechip expression 3' amplification one cycle Target Labelling and Control Agents. In detail the first strand cDNA was synthesized using 5µg whole RNA sample by superscript 11

reverse transcriptase. The synthesis of second strand was done as strand replacement reaction using the *E.coli* DNA polymerase I complex, hybridstrand specific RNA degrading RNAase H, a Ligase reaction (e.coli DNA ligase) and finally an end polishing with recombinant T4-polymerase was performed. Biotin-16-UTP was introduced as label by a linear amplifying i*n vitro* transcription reaction using T7 polymerase overnight 16 h. The required amount of cRNA was fragmented by controlled chemical hydrolysis to release the proportionality of cRNA molecule length and the amount of incorporated biotin derivative. The hybridization was carried out overnight 16 h at 45ºC in the GeneChip Hybridisation Oven 640 (Afymetrix). Washing and staining after hybridization were performed with the Affymetrix Fluidics Station 450. In order to achieve a signal enhancement, an antibody amplification was carried out using a biotinylated anti-streptavidin antibody (Vector Laboartories, U.K), which was cross linked by goat IgG (Sigma) followed by a second staining with streptavidin-phycoerythrin conjugate (Molecular probes, Invitrogen). The scanning of the microarray was done with GeneChip scanner 3000 (Affymetrix) at 15.6 micron resolution. The data analysis was performed with the MAS5.0 (Microarray suite statistical algorithm, Affymetrix) probe level analaysis using Genechip Operating Software (GCOS 1.4) and the final data extraction was done with the data mining tool 3.1 (Affymetrix).

2.7.12.1. Heat map view and gene ontology analysis

The functional and biological classification of the differentially regulated genes of the microarray data was done by using the PANTHER classification system. PANTHER is a browsable database of gene products with organized annotation for the overall biological functions and the pathways involved with gene product (Thomas *et al.*, 2003). The heat maps were generated based on the microarray data by using the TM4 a free, open-source system for microarray data management and analysis (Saeed *et al.*, 2003). We have used the hierarchical clustering to cluster the differentially expressed genes at the various timepoints 0,15,30,60 and 180 minutes. The expression profiles on the heat map were represented on 2 fold change scale.

2.7.13. Validation of microarray results by quantitative real time PCR

2.7.13.1. RNA isolation and cDNA synthesis

Total RNA was isolated with the RNeasy RNA isolation kit according to the manufaturer's advice as mentioned in (2.7.11.6). The reaction mixture for reverse transcription of RNA contained 5 mM $MgCl_2$, 10 mM each of dATP, dGTP, dCTP and dTTP, 25 U Avian

Myoblastosis Virus Reverse Transcriptase (AMV RT), 0.6 µg oligo (dT15) primer, 0.6 µg oligo dt primer and 1 µg total RNA in 50 µl of PAN Script NH4 buffer. The mixture was further heated upto 42°C, incubated for 1 h, inactivated by heating to 95°C for 5 min and the samples were stored at -20°C.

2.7.13.2. Quantitative real-time PCR

Real-time PCR was performed with the Applied biosystems PCR mix. The reaction mixture contained 3 mM MgCl2, 0.2 µM of each primer and 1 µl of cDNA. PCR was carried out in the Applied Biosystems AB 7900 over 45 cycles under the following conditions: denaturation step at 95°C for 10 s, step-down annealing (67°-57°C, 1°/cycle) over 10 cycles and constant annealing temperature of 57°C thereafter for 10 s, elongation at 72°C for 12 s. The following Taqman specific assay probes were used for the assay Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control against which all samples were normalized.

Table 1: Details of the Taqman assays specific for human genes

Target	**TaqMan Assay ID**
PIP5K1A	Hs00740299_mH
SNX12	Hs00362725_m1
KIFC1	Hs00382565_m1
LMNB1	Hs00194369_m1
GAPDH	Hs4392938

2.7.14. siRNA mediated knock down of the host genes

2.7.14.1. siRNAs

Silencer Select siRNAS from Applied Biosystems have been verified experimentally by the manufacturer to reduce the expression of their target genes by ≥80% in at least 3 biological replicates. We have used siRNAs for the host genes inositol polyphosphate phosphatase-like 1 (INPPL1), kinesin family member C1 (KIFC1), phosphatidylinositol-4-phosphate 5-kinase, type I, alpha (PIP5K1A), Cytoplasmic dynein heavy chain 1 (DYNC1H1) were purchased from Applied Biosystems. The siRNAs which target the host gene guanine nucleotide binding protein (G protein), gamma 12 (GNG12), the non targeting negative control were purchased

from Qiagen and the siRNA for low density lipoprotein receptor adaptor protein 1 (LDLRAP1) was purchased from Invitrogen. The details of the siRNA are mentioned in the table 2.

Table 2: Details of the siRNA used for the host gene silencing studies

Target	Sense strand	Antisense strand
INPPL1	CAAUCAUGUGGAAUAUCAtt	UGAUAUUCCACAGUGAUUGca
PIP5K1A	GGCUCAACCUACAAACGGCtt	GCCGUUUGUAGGUUGAGCCtt
KIFC1	CCUCAACUCUCUACGCUUUtt	AAAGCGUAGAGAGAGGga
GNG12	CGAUAUGUCAGGACCUAAATT	UUUAGGUCCUGACAUAUCGGA
DYNC1H1	GGAGCGAAUGAAUACCCUUtt	AAGGGUAUUCAUUCGCUCCag
Negative Control siRNA	UUCUCCGAACGUGUCACGUdTdt	ACGUGACACGUUCGGAGAAdTdT
LDLRAP1	UGUCCAGUUCUCAGGCAGC	GCUGCCUGAGAACUGGACA

2.7.14.2. Reverse transfection of siRNA into Huh7 cells

Huh7 cells were reverse transfected using the commercially available Lipofectamine RNAi MAX transfection reagent. Briefly, 5 nM of siRNA was mixed in 500 µl of Opti-MEM I medium without serum was added to each well of the tissue culture plate. The Lipofectamine RNAiMAX transfection reagent was gently mixed on a vortex mixer and added 3 µl to each well. The transfection complexes were mixed gently and incubated at room temperature for 20 minutes. Huh7 cells diluted in complte growth medium without the addition of antibiotics were added to each well at 0.2- 0.3 million cells per well. The cells were incubated at 37°C for 24 hours.

2.7.14.3. Infection on Huh7 cells using HCVcc

After 24 hours of transfection the Huh7 cells were infected with HCVcc as mentioned in (2.7.11.5).

2.7.14.4. Quantitative real-time PCR assessment of Knock down efficiency

Knock down efficiency of the target genes after 48 hours of infection, were verified by q-PCR method. RNA was isolated and cDNA synthesis was done as mentioned in (2.7.11.6) and (2.7.13.1). Taqman specific assay for PIP5K1A, KIFC1, GNG12 were used and the assay was

performed as mentioned in (2.7.13.2). Hepatitis C virus RNA levels were quantified using the JFH1 specific taqman probes and the assay was performed similarly as mentioned above. The expresion levels of DYNC1H1 were evaluated using the Syber Green based real time PCR. The quantitative reverse transcription-PCR (qRT-PCR) was done using a Quantitect SYBR green PCR kit (Qiagen) with 0.2 µM of each primer and 1 µl of cDNA. Primers used for DYNC1H1 and GAPDH are mentioned in the Table. The fold change was calculated based on the comparative threshold cycle (CT) method. (GAPDH) was run as an endogenous control against which all samples were normalized. The details of the primer sequences are mentioned in the table 3

Table 3: Details of the primer

Target	Forward primer	Reverse primer
DYNC1H1	CAAUCAUGUGGAAUAUCAtt	UGAUAUUCCACAGUGAUUGca
GAPDH	GGCUCAACCUACAAACGGCtt	GCCGUUUGUAGGUUGAGCCtt

2.7.15. Cell viability assay

The cell viability of siRNA treated cells was measured by using the colorimetric WST-1Asaay (Roche), according to the manufactures instructions. The assay principle states that tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an incease in the amount of fromazan dye formed which directly correlates to the number of metabolically active cells in the culture and the adsorbance of the dye solution measured at 420- 480nm. Briefly, 10 µl of the cell proliferation agent WST1 was added to each well of the 96 well format cell culture dish and the cells were incubated at 37°C for 1 hour. After incubation the plate was gently shaked in the ELISA reader and the adbsorbance were measured at 420- 480 nm.

2.8. Expression analysis of HCV-NS4B, using adenovirus expression system

2.8.1. Construction of recombinant adenovirus encoding HCV NS4B

Recombinant adenovirus was constructed using the AdEasy system (Stratagene) (He *et al.*, 1998). Briefly, HCV NS4B was amplified by PCR from the full length HCV NS4B CON1 pET32a-His Trx.and cloned into the pAdTrack-CMV shuttle plasmid using BglII and SalI.restriction sites according to the methodology of the pAdEasy-1 system (Fig. 4) (He *et al.*,

1998).The primers used for the cloning is listed in the table 4. Sequence verification of the clones was done by sequencing in both forward and reverse directions. The pAdEasy system uses homologous recombination in the recA+ E.coli strain BJ5183 to introduce the gene of interest HCV NS4B into the adenovirus background. Shuttle vector pAdTrack-CMV-NS4B was linearized with *pme I* and electroporated to BJ5183 *E.coli* cells. The recombinats were selected on the basis of the Kanamycin resistance and this was further confirmed by restriction digestion using pme1 & pac1.

Table 4: Details of the primer

Forward	**5' AGATCT ATGGCCTCACACCTCCCTTACATCG 3'**
Reverse	**5' GTCGACTAGGCATGGCGTGGAGCAGTCCTCGT 3"**

2.8.1.1. Production of adenovirus

PacI-digested recombinant adenoviral DNA (4 µg) was transfected in HEK293 cells in T-25 flask using Turbofect (Fermentas) according to manufacture instructions. Transfected cells in DMEM medium containing 10% FCS were monitored for cytopathic effects (CPE), which were evident by approximately 6 to 10 days post-transfection. Cells were harvested and freze thawed for 3 cycles in dry ice and at 37°C water bath to release the virions from the cells. The supernatant was stored at -80°C as aliquots untill use.

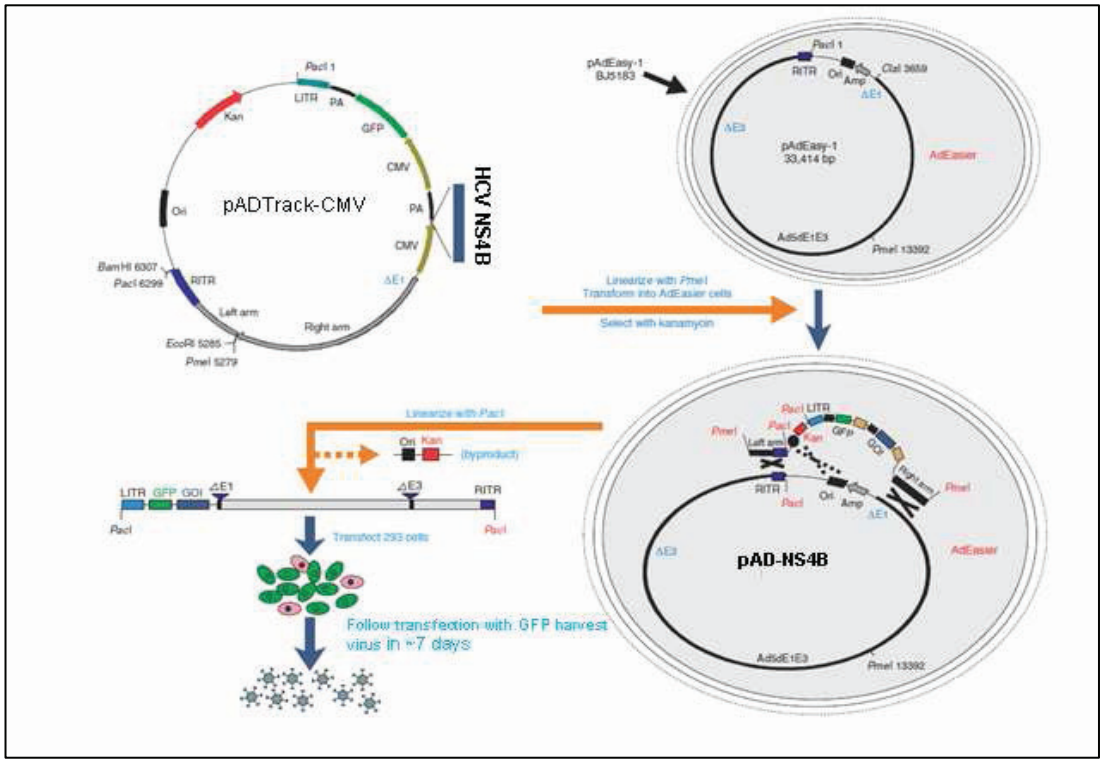

Figure 4: Schematic representation of the AdEasy technology. The gene of interest (here HCV-NS4B) is first cloned into a pAdTrack-CMV. PmeI linearized plasmid is transformed into competent AdEasier cells, which are BJ5183 derivatives containing the adenoviral backbone plasmid pAdEasy-1. Kanamycin resistant recombinants are selected and confirmed by restriction endonuclease analyses. The confirmed recombinant adenovirus plasmids are digested with PacI to generate both inverted terminal repeats (ITRs) and transfected into HEK-293 cells which express recombinant adenovirus E1, allowing them to produce adenoviruses from backbone vectors without the E1 gene. The 'left arm' and 'right arm' are responsible for the homologous recombination between the shuttle vector and the adenoviral backbone vector. The dotted line represents the alternative homologous recombination between two Ori sites. LITR: left-hand ITR and packaging signal, PA: polyadenylation site;; RITR: right-hand ITR. (He *et al.*, 1998; Luo *et al.*, 2007). (Adapted with permission from Nature publishing group, License Number: 2674991352501)

2.8.1.2. Propagation of recombinant adenovirus

HEK293 cells were plated in 25-cm^2 tissue culture flasks (Nunc) at 80–90% confluency (approximately 3 × 10^6 cells per flask in 7 ml complete DMEM) 6–15 h before infection. HEK293 cells were infected by adding 40–50% of the primary transfection viral supernatants (i.e., 0.5–1.0 ml of the 2.0 ml viral lysate) to each 25-cm^2 flask. The lysates from the primary

transfection used in each infection were determined by their initial titers (usually in the range of 10^6 – 10^8 infectious particles per ml). The rest of the viral lysate were stored at -80°C. A CPE or cell lysis by adenoviral vectors became evident at 2–3 days after infection. Productive infections were easily observed by detecting GFP by fluorescence microscopy. When most of cells become rounded up and about half of the cells were detatched (3 to 4 days post infection) cells were harvested and stored at -80°C until use.

2.8.1.3. CsCl gradient purification of Adenovirus

The stored cell pellets were thawed and resuspended in 15 ml of 0.1M Tris in 50ml falcon tubes. To the resuspended cell pellet, 1.5ml of 5% sodium deoxycholate was added, mixed thoroughly and kept at room temperature for 30 minutes until the solution became clear and viscous. After the incubation, 150 µl of MgCl2 and 75 µl of DNase1 solution (5mg/ml in EtOH) was added and incubated at 37°C for 1 hour (Mixed at 10 minute interval) until the solution become less viscous. The gradient was prepared on ice. Now the virus solution was centrifuged at 4000 rpm for 15 minutes at 4°C. Then 5 ml of the supernatant was poured with most care into the gradient tubes and centrifuged at 35000 rpm for 1 hour at 10°C. After centrifugation the three bands were collected using the syringe and transffered into the 1.35 density solution, mixed thoroughly and centruged at 35000 rpm for 24 hours at 10°C. After the final centrifugation ends, there was only one visible band which was collected using the syringe as mentioned above. The purified virus band was dialyzed using the slide a lyzer dialysis chamber (PIERCE). Before the addition of virus into the chamber the excess air in the chamber was removed using the syringe. The purified virus band was added to chamber and dialyzed for 24 hour at 4°C in pre-cooled 10mM Tris/HCl. After 24 hours the virus from the chamber was removed and diluted with 50% glycerol in PBS to a final concentration of 10%. The OD of the virus stock was measured using the quartz cuvette at 260 nm and stored at -80°C.The viral yield was calculated as 1 O.D at 260 is equals to 10^{10} viruses.

2.8.1.4. Protein assay (Bradford assay)

The assay was performed using the commercially available Bio-Rad Bradford dye reagent concentrate. To the diluted reagent (1:6 in water) 10 µl of the sample to yield a final volume of 1 ml, and incubated at RT for 5 min. Afterwards, 400 µl of the reaction mixture were transferred to a cuvette and the absorbance was read at a wavelength of 595 nm in Biophotometer. The protein concentrations were calculated based on the standard curve prepared with BSA ranging from 5 to 50 µg.

2.8.1.5. SDS-PAGE

Solutions

Acrylamide solution: 29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (Sigma)

SDS: 10% (w/v)

APS solution: 10% (w/v)

TEMED

Running gel buffer: 0.5 M Tris / HCl, pH 8.8

Stacking gel buffer: 1.5 M Tris /HCl, pH 6.8

Electrode buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS

Five-fold concentrated sample buffer: 0.16 M Tris / HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 0.38 M mercaptoethanol, 0.008% (w/v) bromophenol blue; pH 6.8

Preparation of the gels

The gel size was 8 cm x 10 cm x 1 mm. The stacking gels were prepared with 3-5% acrylamide at pH 6.8, the running gels with 10% acrylamide at pH 8.9. The scheme as shown in table 5 was used for casting the gels:

Table.5: Scheme for casting SDS-PAGE gel.

Solution	10% running gel	3% stacking gel
30% Acrylamide solution	3.33 ml	0.50 ml
Running gel buffer	2.5 ml	
Stacking gel buffer		1.25 ml
Water	4.05 ml	3.0 ml
10% (w/v) SDS	100 µl	50 µl
TEMED	20 µl	5 µl
10% (w/v) APS solution	37.5 µl	200 µl

Preparation of the samples

Protein solution was mixed with 5-fold concentrated sample buffer and filled up with ddH_2O to yield a final concentration of 1-fold sample buffer in a maximal volume of 18 µl. The mixture was then heated to 95 °C for 7 min. After collecting the condensate by brief centrifugation, the samples were applied to the gel.

Electrophoresis

Electrophoresis was performed at constant current (20 mA) at RT. When the bromophenol blue front had reached the end of the running gel, the process was stopped. The gels were either stained with Coomassie Brilliant Blue R 250 or used for Western blotting.

2.8.2. Western blot analysis with chemiluminescence detection

2.8.2.1. Experimental procedure

Proteins (20 µg) were separated by discontinuous SDS PAGE. The protein bands were then transferred from the gel to a nitrocellulose membrane as detailed below. The nitrocellulose membrane was rinsed with transfer buffer. The transfer "sandwich" was packed by piling a plastic lattice, a synthetic fiber mat, a filter paper, the SDS polyacrylamide gel, the nitrocellulose membrane, a filter paper, a synthetic fiber mat and a final plastic lattice. Air bubbles were strictly avoided in the process. The arrangement was inserted into an electroblot chamber filled with transfer buffer. Electrophoretic protein transfer was performed with a current of 140 mA at 4 °C for 2 h. After transfer, the membrane was removed from the transfer chamber and stained by brief immersion in Ponceau S solution. Subsequently the membrane was destained with 0.05% (v/v) Tween 20 / PBS, washed with PBS and processed as described in the following.

Protein transfer solutions

Transfer buffer: 25 mM Tris / HCl, 192 mM glycine; pH 9.0

Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid

2.8.2.2. Detection of protein bands with the enhanced chemiluminescence (ECL) reagent

The membrane was incubated overnight with 20 ml blocking solution at RT in order to block unspecific binding sites. It was then incubated with appropriate antibodies. The incubation time was 1 h. The membrane was washed three times for 5 min with 20 ml TBST, followed by incubation with 20 ml of the secondary antibody solution for 1 hour to overnight. The membrane was washed again three times for 5 min with 20 ml TBST, rinsed briefly with PBS and then the ECL detection solution was added dropwise to cover the membrane. After 2 min, the solution was removed and the membrane wrapped in transparent plastic foil. An X-ray film was exposed to the membrane for approximately 5 min in an exposure cassette. The film was developed in an X-ray film developing machine.

Solutions

Washing buffer: 20 mM Tris / HCl, 150 mM NaCl, 0.02% (v/v) Tween 20; pH 7.4 (PBST)

Blocking solution: 5% milk powder in PBST

Substrate solution: 250 µl ECL solution I + 6 µl ECL solution II (Amersham)

2.8.3. Proteome profiler Phospho Kinase array

2.8.3.1. Sample prepration

The sample preparations were done according to manufactures instructions. The cells were rinsed with PBS and solubilized in lysis buffer and the fully resuspened extract were gently shaked for 30 minutes at 4°C. Microcentrifuge was done at 14000 g for 5 minutes and the supernatant was transferred to a clean tube. The total protein assay was done as mentioned in (2.8.1.4).

2.8.3.2. Reagent prepration

The phospho kinase array contains eight nitrocellulose memebranes which are designated as part A and part B, in which the part A was spotted with 28 antibodies and part B with 18 antibodies in duplicate. Part A and Part B were used together for the optimal analysis efficiency. The detection antibody cocktails A and B were reconstituted with 100 µl of water. The rest of the reagents were ready to use.

2.8.3.3. Array protocol

The human phospho kinase array part A and part B was incubated with 1ml of array buffer in separate wells of the 8 well multi dish at room temperature for I hour which served as a initial blocking step. After blocking the array buffer was removed and diluted 2 ml of cell extract was added to each well and incubated overnight at 4°C on a rocking platform. After overnight incubation the membranes were removed and washed three times in seprate dishes with 1x wash buffer. After washing, 20 µl of reconstituted antibody cocktails A and B was added to both the membranes. The membranes were incubated at room temperature for 2 hours on a rocking platform. The membranes were washed after 2 hours with 1x wash buffer. Diluted streptavidin –HRP in 1x array buffer was added to each well. The excess wash buffer was drained from the membranes and were returned the membranes to the 8 well dish and incubated with streptavidin–HRP for 30 minutes at room temperature. After incubation, the memebranes were washed 3 times in the 1x wash buffer for 10 minutes. The excess buffer was

drained from the membranes part A and B. Both the membranes were exposed and developed using the chemiluminescent reagents as mentioned in (2.8.2.2)

2.8.3.4. Data Analaysis

To identify the positive signals on the developed film was aligned on the array image using three pairs of positive control spots in the corners of each membrane (two pairs on the left side of Part A and one pair on the right side of Part B). The stamped identification numbers on the membranes were placed on the left hand side. Location of the controls and capture antibodies were identified as mentioned in the appendix supplied by the manufacturer booklet. Signal intensities were quantified using the TINA software and normalized with the positive controls.

2.8.4. Microarray

Huh7 cells were transduced at a multiplicity of infection (MOI) of 20 with either adenovirus expressing HCV NS4B (Ad-NS4B) or an adenovirus with green fluorescent protein (Ad-GFP) which served as a control. Total cellular RNA was extracted 60 hours post-infection with an RNeasy Mega kit (Qiagen) as mentioned in (2.7.11.6). RNA samples were purified; quality tested using an agilent bioanalyzer and hybridized on to Human Genome U133 plus 2.0 array (Affymetrix) for fluorescence data acquisition as mentioned in (2.7.12). The detailed analysis of the microarray data was done as mentioned in (2.7.12).

2.8.4.1. Quantitative real-time PCR validation of microarray results

qRT-PCR quantification of the host gene expression using all of the RNA samples from Ad-HCV NS4B versus Ad-GFP at 60 hpt was performed for ARG1. Real-time PCR was performed with the QIAGEN Syber green PCR mix. The reaction mixture contained 3 mM MgCl2, 0.2 µM of each primer and 1 µl of cDNA. PCR was carried out in the Applied biosytems 7900 over 45 cycles under the following conditions: denaturation at 95°C for 10 s, step-down annealing (67°-57°C; 1°/cycle) over 10 cycles and constant annealing temperature of 57°C thereafter for 10 s, elongation at 72°C for 12 s. The following gene specific primers were used .GAPDH was used as an endogenous control. Details of the primers used for the study is mentioned in the table 6.

Table 6: Details of the primer

Gene name	Forward Primer	Reverse primer
ALB	AAGCTGCCTGTTGCCAAA	TCAGGCGAGCTACTGCCCATGC
PEX16	GCCGGACCATCCTGCTGCTCTA	AAGTAATCCATGAGCGGCCTTGTG

ARG1	CTCAAAGGGACAGCCACGAGGA	GATGTCAGCAAAGGGCAGGTCCC

3. Results

3.1. Global modulation of gene-expression changes during the synchronized infection of HCVcc on Huh7 cells

The developemnt of an infectious cell culture system and retrovirus-based pseudoparticles with HCV envelope glycoproteins (HCVpp) (Bartosch *et al.*, 2003a) (Wakita *et al.*, 2005) was a breakthrough for HCV research. The HCVcc system enabled *in vitro* studies on various aspects of the virus life cycle. Even though we have the HCVcc infectious system, synchronization of the infection is more important to study the kinetics of virus entry and the subsequent viral and cellular events. Due to this we used the synchronized HCV cc system developed by Dr. Aravind Patel's lab at MRC virology, Glasgow. The synchronized HCVcc infections were completely done at MRC virology, Glasgow. HCV entry is a multi step entry process with the involvement of many host cell receptors and following intracellular events. The main objective of our study was intended to screen the transcriptional changes during the entry process of HCV infection. Because of this we decided to choose early time points at 0, 15, 30, 60, and 180 minutes for the microarray study. The synchronized infection of HCVcc on Huh7 cells was verified by immunofluorescence of antibody specific for HCV NS5a after 48 hours (Fig. 5).

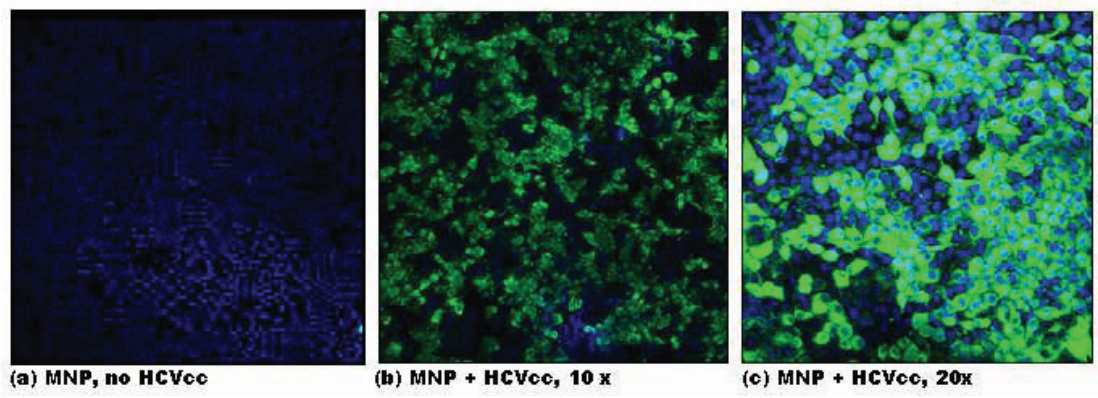

Figure 5: Infection of Huh7 cells after synchronization of HCVcc attachment to cells by magnetic nanoparticles (MNPs).(a) Cells were treated with MNPs and no HCVcc (b) after 48 hours cells were washed and fixed with methanol and probed for HCV non structural protein 5A (NS5A-Green).Nuceli stained with DAPI (Blue).10x magnification (c) 20x magnification.

We have used the Affymetrix HGU133 plus 2.0 genechip arrays to compare the HCV infection in combination with combi mag magnetic particles or with combi mag particles alone in Huh7 cells. The total RNA was isolated at the indicated timepoints from 0 to 180 minutes and this

was used for the microarray studies. The microarray data were analyzed using the Affymetrix MAS 5 algorithm and the summary of the genes that were up or down-regulated are shown in table 7.

Table 7: List of Up-regulated and down-regulated genes in response to the synchronized infection of HCVcc with magnetic particles on Huh7 cells.

Affymetrix ID	Gene bank accession no:	Gene symbol	Gene name	Fold change
Up-regulated genes				
209680_s_at	NM_002263.3	KIFC1	kinesin family member C1	1.7
232233_at	NM_033125.2	SLC22A16	Solute carrier family 22 (organic cation/carnitine transporter) member 16	1.6
231723_at	NM_013346.2	SNX12	Sorting nexin 12	2.0
205010_at	NM_001184819.1	GNL3L	Guanine nucleotide binding protein-like 3	1.6
210256_s_at	NM_001135637.1	PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha ,transcript variant 4	1.6
229115_at	NM_001376.4	DYNC1H1	Dynein, cytoplasmic 1, heavy chain 1	1.8
217427_s_at	NM_003325.3	HIRA	Histone cell cycle regulation defective homolog A (S. cerevisiae)	1.8
222026_at	NM_006743.3	RBM3	RNA binding motif protein 3	1.7
205195_at	NM_001283.3	AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	1.6
203276_at	NM_005573.3	LMNB1	Lamin B1, transcript variant 1	1.9
205196_s_at	NM_001283.3	AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	1.6
222795_s_at	NM_018390.3	PLCXD1	Phosphatidylinositol-specific	1.9

			phospholipase C, X domain containing 1	
Down-regulated genes				
201602_s_at	NM_001143885.1	PPP1R12A	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	-1.5
200637_s_at	NM_002840.3	PTPRF	Protein tyrosine phosphatase, receptor type, F	-1.5
224568_x_at	NR_002819.2	MALAT1	Metastasis associated lung adenocarcinoma transcript 1	-1.7

As displayed in (Fig. 6) most of the genes were differentially expressed at 30 minutes post infection. Heat maps of the upregualted genes were generated using the multi experiment viewer and the expression values were represented on a range of -2 to +2 fold change.

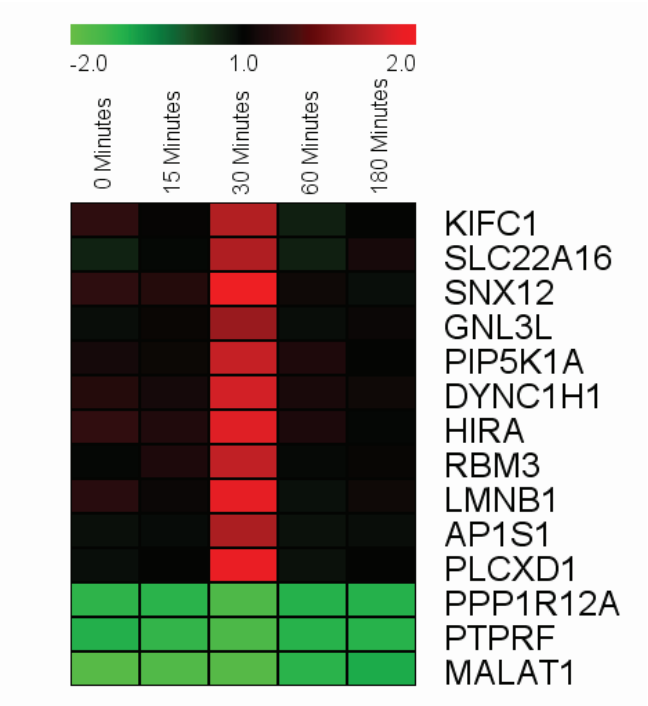

Figure 6: Heat map of differentially expressed genes regulated by synchronized JFH1 infection. Fold changes were calculated by comparing gene expression in JFH1 infected Huh7 cells to that of mock infected Huh7 cells at 0, 15, 30, 60 and 180 minutes post infection. Expression profiles represented on a range of -2 to +2 fold change. Red denotes the up-regulated genes and green denotes the down-regulated values as compared by the median value. Heat maps were generated using Multi Experiment Viewing Software (Mev).

Gene ontology analysis was done using using PANTHER software revealed that that a significant proportion of differentially expressed genes were associated with vesicle mediated transport, signal transduction and endocytosis (Fig. 6).

3.2. Quantitative real-time PCR validation of microarray results

Microarray results showed an HCVcc specific upregulation of 10 genes at the earliest timepoint 30 minutes with a fold change from 1.5 to 2 fold. Among the up-regulated genelist we have choosed the following genes like KIFC1, PIP5K1A, LMNB1, and SNX12 for the q-PCR analysis using Taqman specific probes. Comparison of magnetic particles alone and HCVcc in combination with magnentic particles treated cells showed an approximately 2 fold upregulation for SNX12 (Fig. 7). There were no specific fold change for the other genes analyzed (Fig. 8).

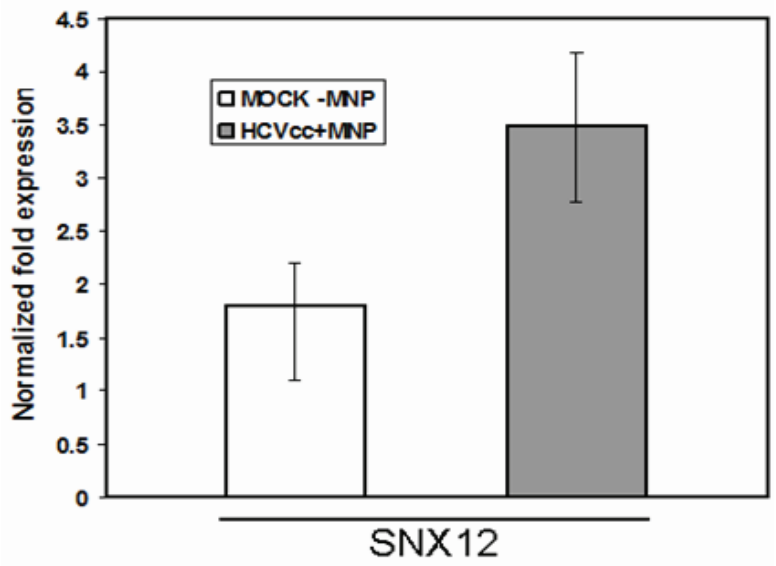

Figure 7: Quantitative real time PCR investigation of microarray results. RNA samples from the 30 minutes timepoint was used for the qRT-PCR analysis. Fold changes in gene expression were calculated for the following genes. (a) Sorting nexin 12 (SNX12) involved in the vesicular trafficking

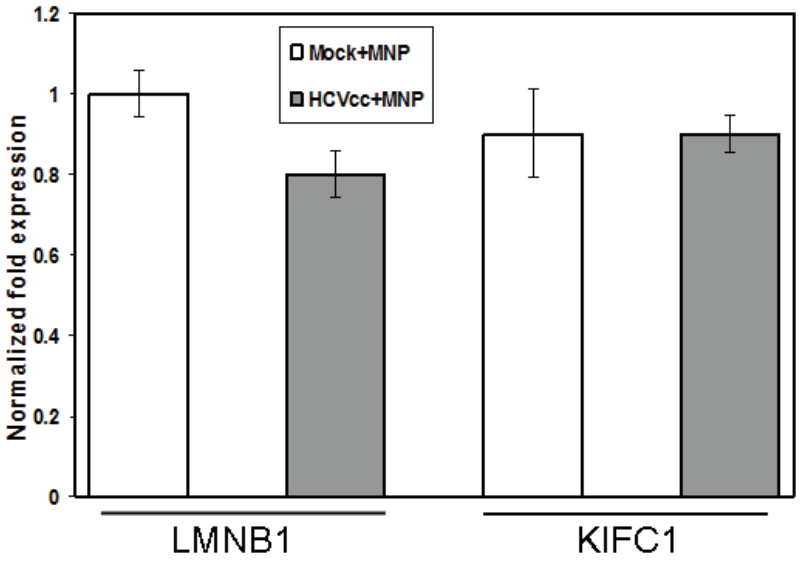

Figure 8: Quantitative real time PCR investigation of microarray results. RNA samples from the 30 minutes timepoint was used for the qRT-PCR analysis. Fold changes in gene expression were calculated for the following genes. Kinesin family member c1 (KIFC1) involved in the movement of endocytic vesicles and lamin b1 (LMNB1) component of nuclear lamina.

3.3. Effect of host gene silencing on HCV infecion

To analyze whether the differentially expressed genes have a direct impact on the efficiency of viral entry, we have selected 6 genes KIFC1, INPPL1, PIP5K1A, LDLRAP1, DYNC1H1, for gene silencing studies. The experimentally validated siRNAS were used for the transient transfection into HUh7 cells by the reverse transfection method. The knock down efficiency of the target genes were verified by the quantification of the mRNA levels using taqman specific probes and syber green based methodologies. Knock down efficiency and JFH1 RNA levels were analyzed after 48 hours post infection In comparison to the non targeting siRNAs, siRNA targeting KIFC1, PIP5K1A, DYNC1H1, were shown to reduce their mRNA levels by approximately 80% (Fig. 9).

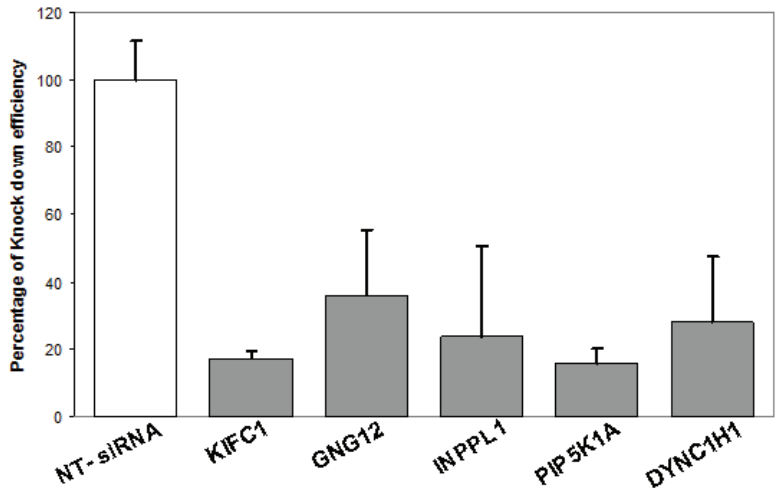

Figure 9: siRNA mediated knockdown of host genes. Knock down efficiency of host genes after 48h post transfection of siRNA

Among the 6 host genes, knockdown of KIFC1 was shown to reduce the infectivity of HCV based on the reduction of JFH1 mRNA levels by approximately 40% (Fig. 10). Cell viability assay showed no toxicity after 48h post transfection of siRNA against the host genes (Fig. 11).

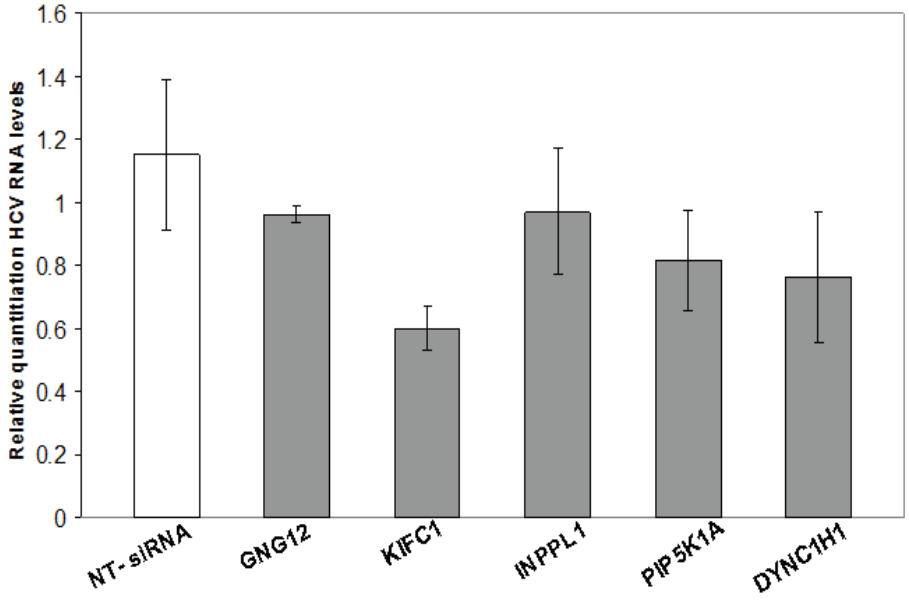

Figure 10: Effect of host gene knockdown on HCV infection 48h post transfection with siRNA molecules targeting host genes were infected with HCVcc. Total RNA was extracted and used in qRT-PCR for quantification of HCV RNA

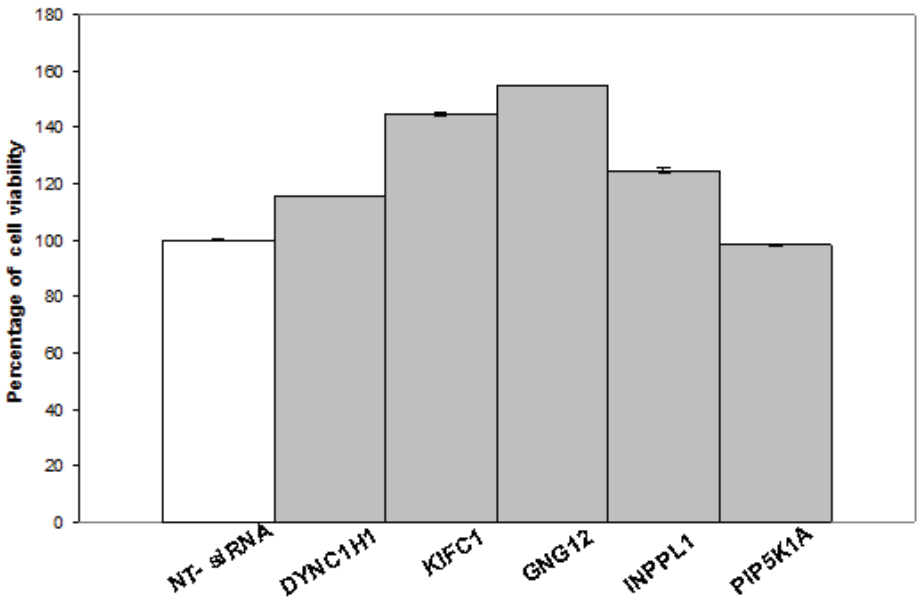

Figure 11: Cell viability of Huh7 cells Cell viability was measured at 48h post transfection.

3.4. Gene ontology analysis of microarray results

We have used the gene ontology program PANTHER (Thomas *et al.*, 2006) to identify the functional classification of the microarray results. PANTHER was used to classify the genelist according to the biological process and cellular component. The genes which were up-regulated or down-regulated at the timepoint 30 minutes with a fold change of -2 to +2 were analyzed by the software. We performed a comparative analysis of our microarray results with the flaviviridae family of viruses like Dengue and Westnile viruses. This analysis was done mainly to uncover hypothetical common features presented by host-Flaviviridae interactions. Based on the literature mining data, the known host factors or genelist for Dengue and Westnile virus infection were collected and the functional classification was done by PANTHER gene ontology program. In comparison with our array results, the functional protein classification of host factors used by Dengue and Westnile virus clearly shows that flaviviridae family of viruses share the common features of host for the infection (Fig. 12). The functional classification based on the biological process clearly showed the majority of the genes were involved in the vesicle mediated transport, signal transduction and endocytosis (Fig. 13 & 14)

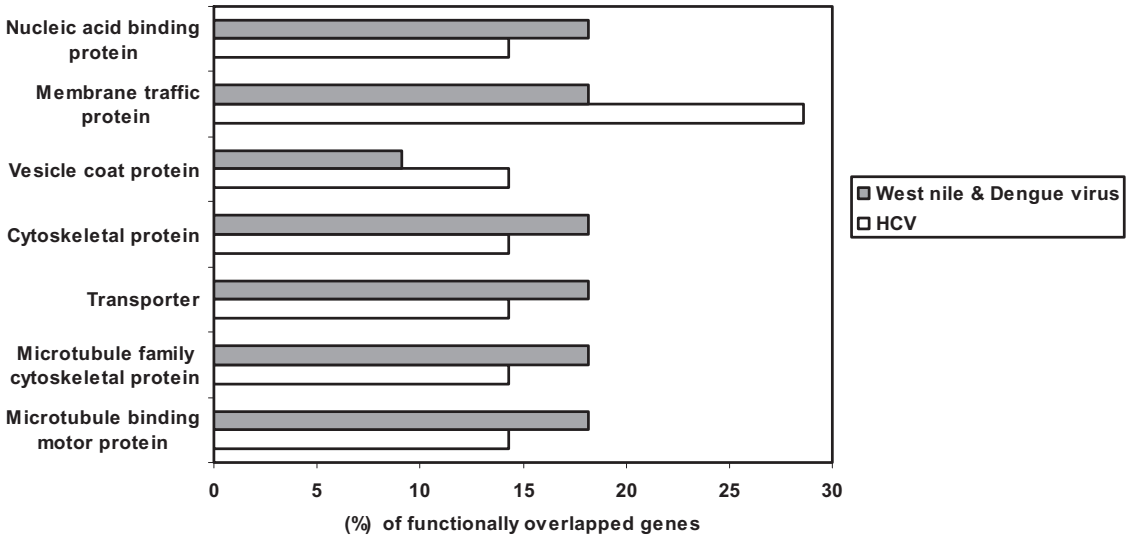

Figure 12: Genes activated by the infection process of West Nile, Dengue and hepatitis C virus.

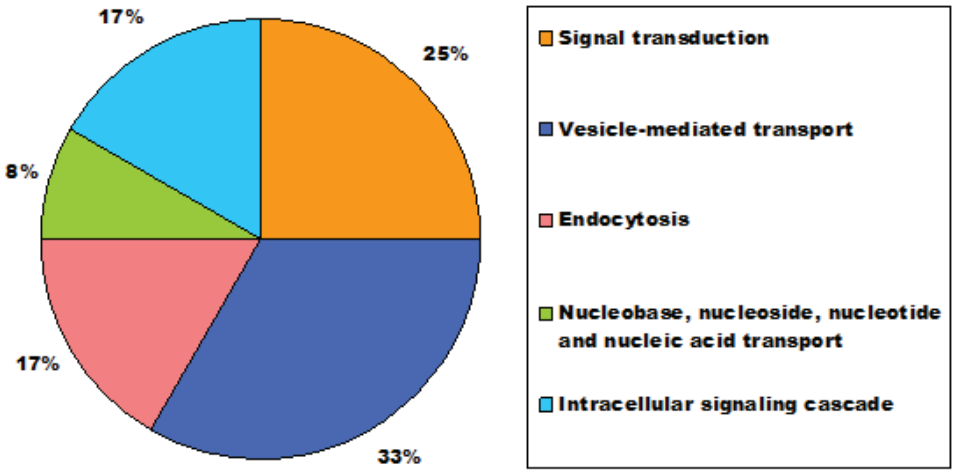

Figure 13: Percentage of up-regulated genes was categorized according to the gene ontology program PANTHER based on the biological process

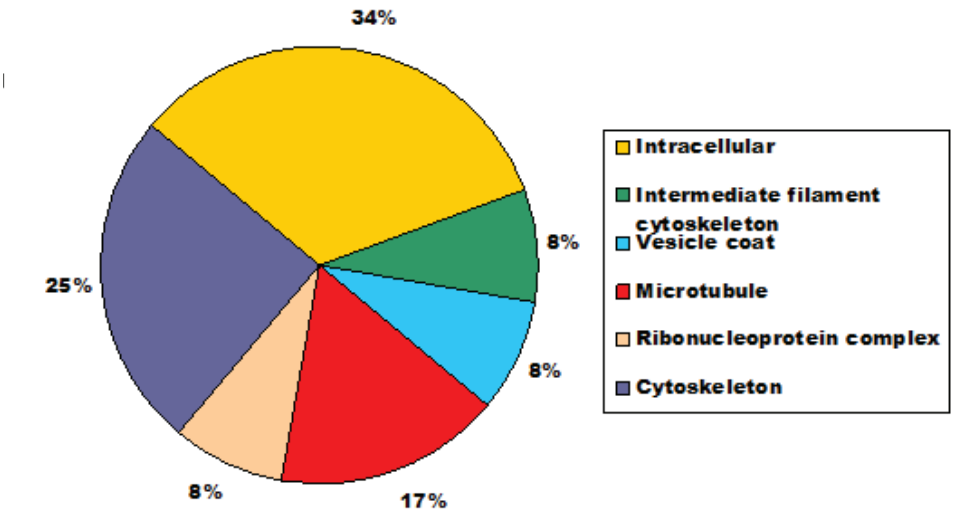

Figure 14: Percentage of up-regulated genes was categorized according to the gene ontology program PANTHER based on the cellular component.

3.5. HCV NS4B adenovirus expression system

HCV NS4B is expected to be translated into a protein of MW 27 kDa. HCV NS4B was expressed using the adenoviral expression system to study the host cellular requirements of this protein in Huh7 cells. The overall strategy of the adenovirus construction is explained in the methods section. HCV NS4B was amplified from the HCV NS4B CON1-pET32a-His Trx and cloned into pAdtrack CMV (Fig. 15).

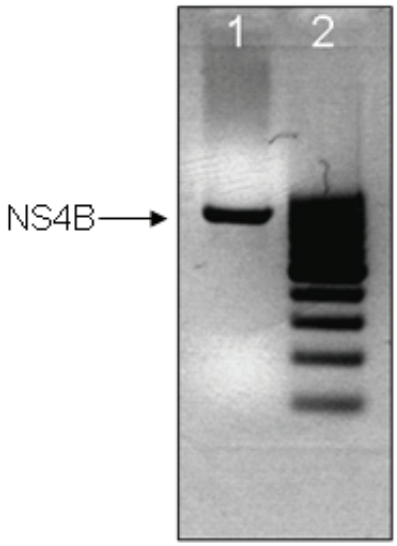

Figure 15: PCR based amplification of NS4B from the HCV NS4B CON1-pET32a-His Trx.Lane (1) NS4B PCR product (2) 100bp DNA ladder.

The resultant construct was linearized with a restriction endonuclease and transformed into E. *coli* strain BJ5183 which harbors supercoiled adenoviral vector pAdEasy-1. Recombinants were selected on the basis of kanamycin resistance and also screened by restriction endonuclease digestion with PacI. Positive clones released a fragment of 4.5kb (Fig. 16) or 3.0 kb to expose its inverted terminal repeats.

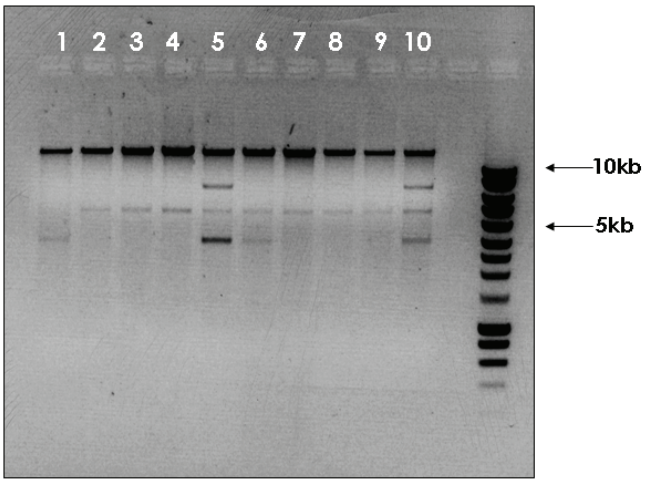

Figure 16: Screenning of Adenovirus recombinants encoding the HCV NS4B. Pac1 restriction endonuclease digestion of recombinants.All but two recombinants (5 and 10) are not considered as positive recombiants. One of the positive recombinant (recombinant number 1) released a 3kb fragment and the rest of the recombinants were released a 4.5kb fragment after

pac1 digestion. Additionally, positevly identified recombinants were also subjected for Con1 NS4B specific colony PCR and confirmed to be positive for the insert NS4B (Fig. 17).

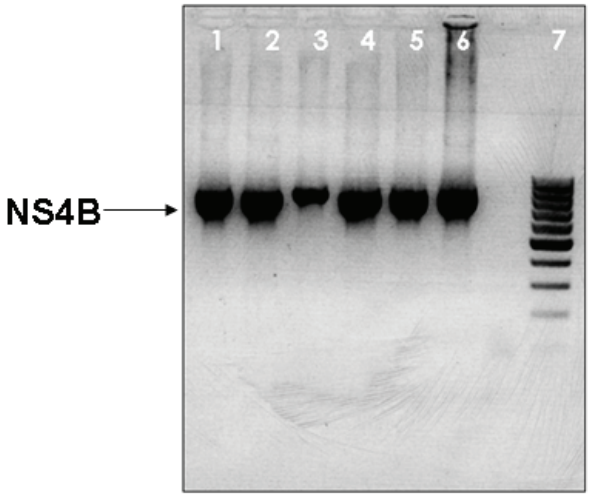

Figure 17: Validation of positive recombinants by colony PCR specific for HCV NS4B. Lane 1 to 5 shows the presence of the HCV NS4B gene. Lane 6 is the PCR product amplified from the HCV NS4B CON1 - pET32a-His Trx as positive control.

The resulting plasmid, pAd-GFP-NS4B, was subsequently transformed to Top10 *E.coli* cells (Invirogen) for large-scale plasmid amplification followed by sequencing using gene specific primers in both forward and reverse directions. The plasmid was re-digested with *Pac*I and transfected into a packaging cell line HEK293 cells. After 7 days, viruses were harvested by freeze thaw cycle and were used for further amplification. Purification of the recombinant adenovius were done by using the CsCl ultra centrifugation and the viral titers were calculated based on the optical density of one OD unit (A260) contains approximately 10^{12} viral particles per ml. The transduction efficiency of the purified adenovirus encoded with NS4B was tested in in hepatoma cell lines like Huh7 with an MOI of 20. Furthermore, we also analyzed the expression levels of NS4B in Huh7 cells at various timepoints. The expression level of HCV-NS4B was detected at 48 and 72 hpt (Fig. 18).

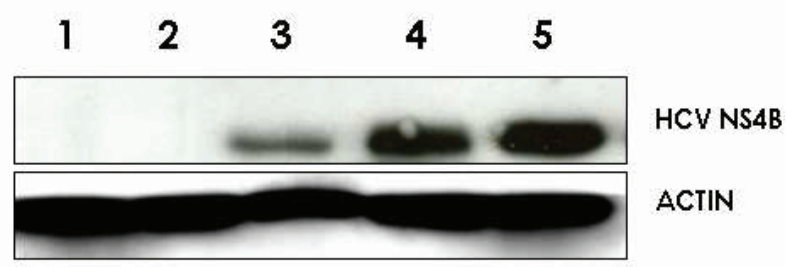

Figure 18: Recombinant adenoviral expression of HCV NS4B in Huh7 cells. Lane (1) Adenovirus encoding green fluorescent protein (Ad-GFP) after 48 hours post infection (2) Ad-GFP after 72 hours of post infection (3) Adenovirus encoding HCV NS4B after 48 hours post infection (4) Ad-NS4B after 72 hours post infection (5) Expression of NS4B protein levels in replicon harbouring stable Huh7 cell lines**.**

3.6. Microarray analysis and Quantitative real-time PCR validation of NS4B expression in Huh7 cells

HCV NS4B has been shown to modulate cellular pathways of host cell to make cellular rearrangements for the formation of replication complex. We have used the Adenovirus encoding HCV NS4B or an Adenovirus encoding the GFP alone to Huh7 cells. The RNA was isolated at 60 hpt and used for hybridization on to Affymetrix HGU133 plus 2 array for fluorescence aquisition. We performed three independent experiments and the fold changes were calculated as log 2 ratios by using the MAS5 algorithm (Affymetrix). The up-regulated and down-regulated genes were described in the table 8.

Table 8: List of up-regulated and down-regulated genes in response to HCV NS4B expression in Huh7 cells

Affymetrix ID	Gene symbol	Gene name	Fold change
Up-regulated genes			
209321_s_at	ADCY3	Adenylyl cyclase 3	4.0
209321_s_at	ARRB1	Arrestin beta-1	3.9
211431_s_at	TYRO3	TYRO3 protein tyrosine kinase	3.7
213418_at	HSPA6	Heat shock 70kDa protein 6 (HSP70B')	3.75
1554264_at	CKAP2	cytoskeleton associated protein 2	3.567

219792_at	AGMAT	Agmatine ureohydrolase (agmatinase)	3.074
217580_x_at	ARL6IP2	ADP-ribosylation factor-like 6 interacting protein 2	2.957
206177_s_at	ARG1	Liver-type arginase	2.6
1566776_at	XLHSRF-1	Dynein, axonemal, heavy polypeptide 1	2.469
230075_at	RAB39B	RAB39B, member RAS oncogene family	2.41
35148_at	TJP3	Tight junction protein 3 (zona occludens 3)	2.406
221604_s_at	PEX16	Peroxisomal biogenesis factor 16	2.2
1555319_at	STAB1	Stabilin 1	2.1
242545_at	TTLL11	Tubulin tyrosine ligase-like family, member 11	2.1
217793_at	RAB11B	RAB11B, member RAS oncogene family	2.032
Down-regulated genes			
233314_at	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	0.25
242568_s_at	MGAT4B	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme B	0.3
1563512_at	CAPON	Nitric oxide synthase 1 (neuronal) adaptor protein	0.2

Microarray results showed that NS4B induced upregulation of host genes like heat shock 70kDa protein 6 (HSPA6), Agmatine ureohydrolase (AGMAT), Type I arginase (ARG1), ALB1.These results show that impact of NS4B potein expression leads to altered transcription of host cell. qRT-PCR experiments were done to validate the expression data obtained from the microarrays. Realt ime qRT-PCR primers specific for HSPA6, AGMAT, ARG1, ALB1, were used and there was 6 fold and 2 fold upregualtion for HSPA6 and. ARG1. HSPA6 are basic

heat unducible forms of HSP70, and ARG1 is known to be involved in the arginine pathway (Fig. 19).

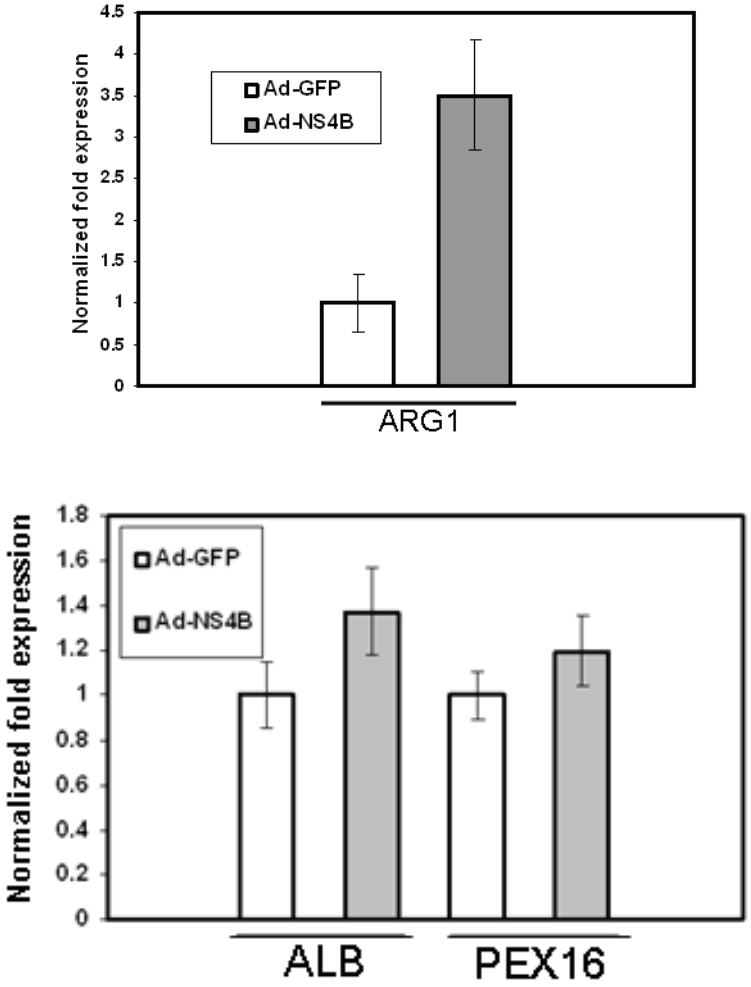

Figure 19: Quantitative real-time PCR investigation of Microarray results RNA samples form the 30 minutes timepoint was used for the q-RT PCR analysis. Fold changes in gene expression were calculated for the following genes. ARG1, ALB1, PEX16.

Functional classification of the up-regulated genes in our microarray study was categorized based on the pathways and molecular function of the corresponding genes (Fig. 18). The majority of the genes were involved in the Wnt signaling pathway, PI3 kinase pathway, Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway, and Arginine biosynthesis pathway.

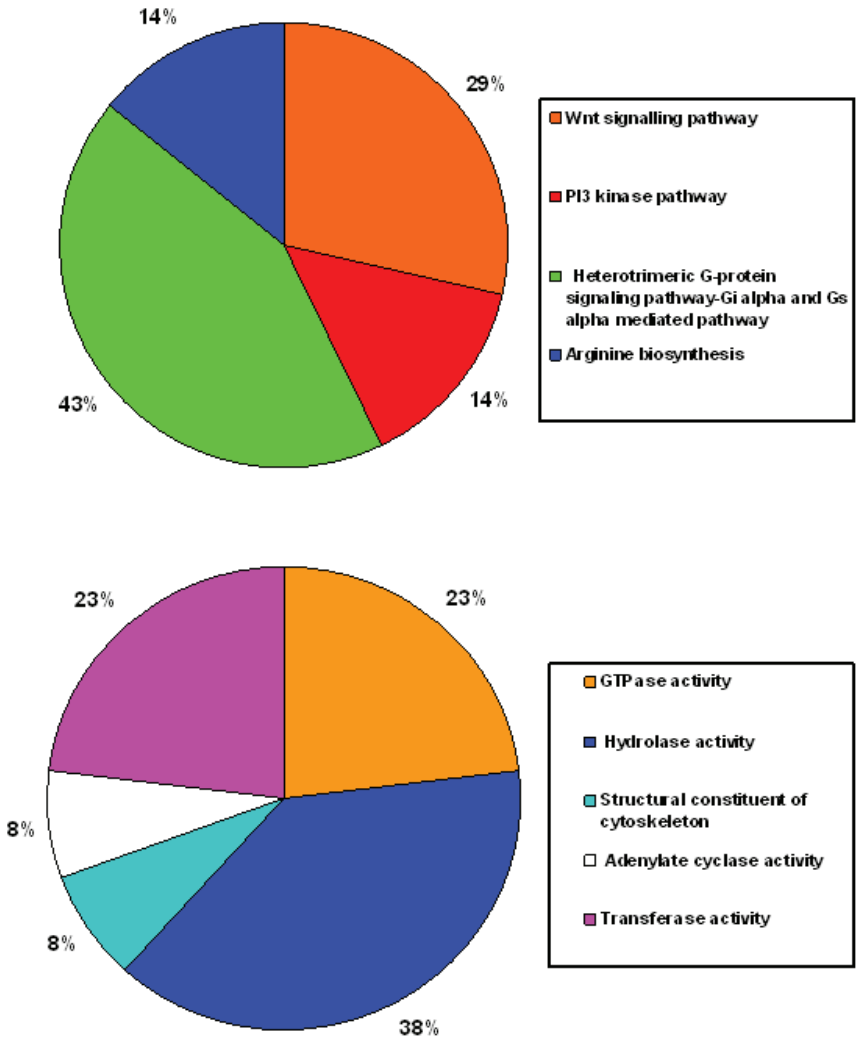

Figure 20: Categorization of genes up-regulated by NS4B according to the gene ontology program PANTHER. (a) Functional classification on the basis of the known Pathways (b) Functional classification of up-regulated genes on the basis of their molecular function of the up-regulated genes**.**

3.7. Phospho-proteome analysis of cellular proteins induced by NS4B

NS4B protein of HCV is well known to modulate the signalling pathways of the host cell. We were interested to analyze the phospho-status of the host proteome by using the phoshoproteome array. The Human Phospho kinase array is a rapid and economical tool to screen the phospho status of 46 kinases spotted on the memebranes. We have performed 2 independent experiments with 4 arrays. The whole cell extracts from the Huh7 cells transduced either with Ad-GFP as a control or with Ad-NS4B was used for the phospho proteome

analysis. Fig. 21 shows the upregulation of cellular kinases upon NS4B protein expression. The total beta catenin levels were up-regulated in addition phopshorylation GSK3 α + ß were also increased in NS4b expressing cells. However the CREB phosphorylation was reduced upon NS4B expression. Intracellular levels of Beta catenin are tightly regulated by the phopshorylation of GSK3 alpha and beta. In the absence of Wnt signal the GSK3 beta will be phosphorylated and this will induce the phosphorylation of beta catenin. Phopshorylation of beta catenin will be directed for proteasomal degradation. Activation of the Wnt signalling pathway will lead to the inhibition of the beta catenin degradative pathway and consequently will increase the accumlation of beta catenin in the cytolasm and nucleus (Barker & Clevers, 2006).

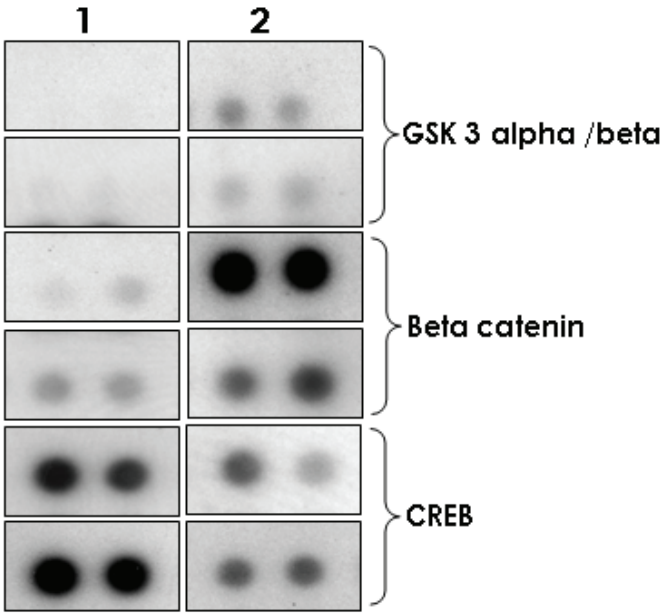

Figure 21: Phospho Kinase proteome profiler array of two independent experiments using Huh7 cells Lane (1) Ad-GFP after 48 hours of post-transduction. Lane (2) Ad-NS4B after 48 hours of post-transduction .The labeled spots are designated as phopshorylated form of GSK 3 alpha/beta at S21/S9, Beta catenin and phopshorylated form of CREB at S133 position respectively.

3.8. Validation of phopsho-array results by western blotting

The differentially regulated proteins identified in the phosphoarray were further validated by Western blotting techniques. We checked the levels of beta catenin and phopshostatus of GSK3alpha at 24 and 48 hpt in Huh 7 cells. The immunoblot clearly showed that NS4B induces the phopshoryaltion of GSK3 and increased the total beta catenin levels (Fig. 22).

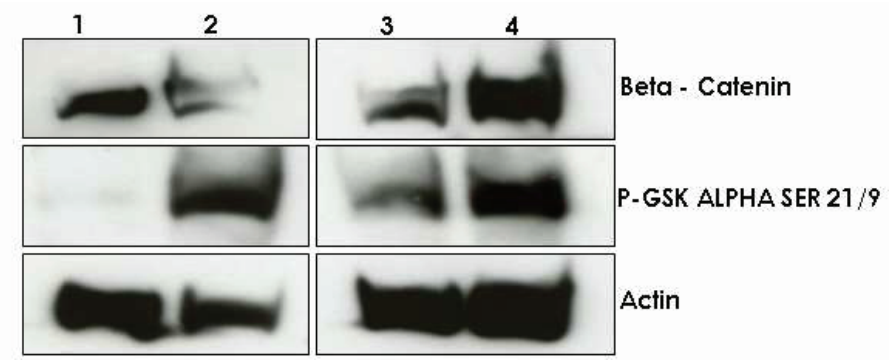

Figure 22: HCV NS4B induces the accumulation of betacatenin in a GSK3 phosphorylation dependent manner Lane (1) Ad-GFP after 24 hours post-transduction Lane (2) Ad-NS4B after 24 hours post-transduction Lane (3) Ad-GFP after 48 hours post-transduction Lane (4) Ad-NS4B after 48 hours of post-transduction.

Next we checked whether the levels of beta catenin in both cytosol and nucleus by nuclear fractionation and western blotting. Actiavtion of Wnt signaling will induce the accumulation and entry of beta catenin into the nucleus and this will further activate transcriptional activation of the TCF promoter. Investigation of beta catenin levels in cellular fractions revealed that beta catenin accumlation was higher in NS4B expressing cells at 90 hours post infection (Fig. 23).

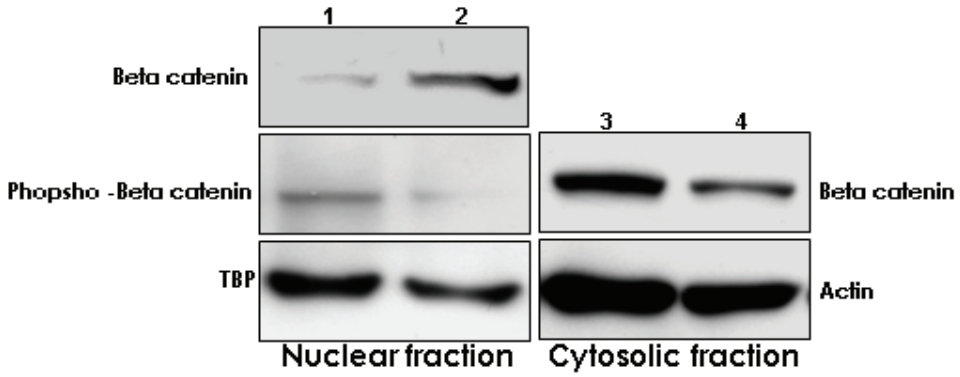

Figure 23: HCV NS4B induces the accumulation of betacatenin in nuclear fraction Lane (1) Ad-GFP after 90 hours post transduction (2) Ad-NS4B after 90 hours post transduction (3) Ad-GFP after 90 hours post transduction (4) Ad-NS4B after 90 hours post transduction.

We have also checked the nuclear levels of beta catenin in HepG2 cells at 85 hpt. As expected beta catenin levels were increased in NS4B transduced cells (Fig. 24).

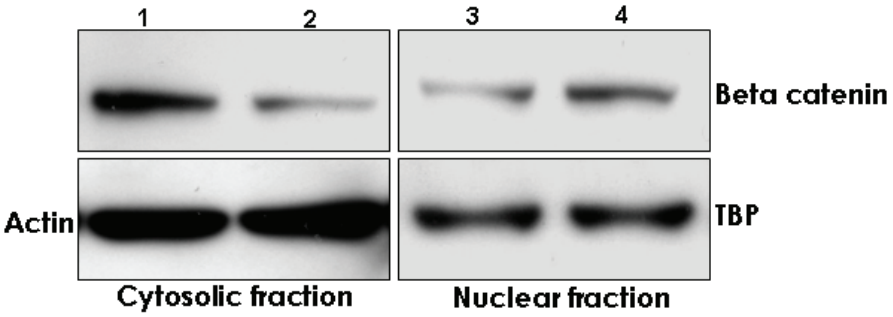

Figure 24: In HepG2 cells, HCV NS4B induces the accumulation of betacatenin in nuclear fraction. Lane (1) Ad-GFP after 90 hours post transduction (2) Ad-NS4B after 90 hours post transduction (3) Ad-GFP after 90 hours post transduction (4) Ad-NS4B after 90 hours post transduction

4. Discussion

4.1. Global modulation of gene-expression changes during the synchronized infection of HCVcc on Huh7 cells

Chronic hepatitis by HCV which is known to affects 170 million people worldwide. New adanced experimental systems were a breakthrough in HCV research. The production of cell culture infectious virus HCVcc has brought a relative simple *in vitro* model (Wakita *et al.*, 2005). Eventhough the infectious system for HCV is available, the synchronization technique of infection was necessary to study the kinetics of viral entry. Synchronized infection in combination with magnetic particles, showed greater efficiency of virus adsorption to the cells, and increased the infectivity rate of cell culture infectious virus, in comparison to other known standard protocols (Vieyres *et al.*, 2009). The synchronized infection system is a necessity to study accurately the kinetics of viral entry and the subsequent intracellular events. Microarray analysis of HCV infected cells can provide an insight into transcriptional changes of the host genes involded in the various phases of HCV life cycle. To better understand the host gene regulation during entry process of HCV, we decided to choose the early time points of synchronized HCV infection on Huh7 cells. We performed the microarray analysis of the synchronized HCVcc infection on Huh7 cells at 0, 15, 30, 60, and 180 minutes. Microaray studies revealed that most of the genes were differentially expressed only at 30 minutes post infection. Functional classifications of our expression analysis data clearly showed that the majority of the genes were associated with vesicle mediated transport, endocytosis and signal transduction. Phosphatidylinositol-4-phosphate 5-kinase type I, alpha (PIP5K1A), one of the up-regulated genes in our study has been already shown to be associated with entry of other viruses like HIV, Dengue and also it is known to inhibit the replication of HCV (Ang *et al.*, 2010) (Barrero-Villar *et al.*, 2008). This protein is a functionally important module in clathrin mediated endocytosis. We also found the gene AP1S1 which is a clathrin adaptor protein involved in clathrin mediated endocytosis. In agreement with our expression analysis data other groups have also found the increased expression of intracellular trafficking genes like sorting nexin 12 (SNX12) during HCV infection on Huh7 cells (Blackham *et al.*, 2010). So, the upregulation of this gene might be important for the entry process of HCV. An increase in the expression of the DYNC1H1 which is known as molecular motor & kinesin family member C1 (KIFC1), which also act as a microtubule dependent motor involved in the movement of early endocytic vesicles. Upregualtion of these genes clearly shows that HCV may require these host

genes for the intracellular movement. Although microarray and HCVcc systems has been used to study the transcriptional changes of host cells during the later stages of HCV infection. However we have used the synchronized HCVcc infection system to investigate global modulation of gene expression signatures at the early time points followed by the binding of HCV to the cell surface receptors.

In this study we found that most of the differentially expressed genes were involved in the intracellular transport and endocytosis. So we decided to knockdown the host genes to investigate its effect on the HCV infection. The genes selected for the knockdown studies were DYNC1H1, KIFC1, PIP5K1A, Guanine nucleotide binding proteins (GNG12). GNG12 is known as a modulator or transducer of various trasnemebrane signaling ystems (Olsen *et al.*, 2006). This gene is mainly involved in the regulation of actin cytoskeleton and MAPK signaling pathway (Weston *et al.*, 2002). Silencing of these host genes in Huh7 cells showed approximately 80% reduction of the mRNA levels in comparison to the non targeting siRNA control. Among these host genes only the silencing of KIFC1 was shown to reduce the infectivity levels of HCV by approximately 40%. There was not much effect on the HCV infectivity rates for the knockdown of the other candidate genes like DYNC1H1, PIP5K1A and GNG12. KIFC1 which is known to be associated with vesicular movement of endocytic vesicles has been shown to affect the infectivity of HCV. This clearly shows that HCV uses the microtubule motor like KIFC1 probably for the intarcellular movement of the virus. Future studies has to be done on KIFC1 to elucidate its molecular mechanism during the HCV infection and this may lead to the discovery of new drug target for HCV antiviral therapy.

4.2. Identification of cellular targets induced by HCV NS4B using microarray analysis

HCV NS4B is known to be the integral memebrane protein which induces cellular rearrangements for the formation of the MW during HCV replication. NS4B codes for a nucleotide binding motiff (NBM) and GTPase activity which control the host cellular pathways. To date, no bonafide cellular targets of NS4B protein to date was largely unknown. Hence we used the adenovirus strategy to express the NS4B protein for the genomic and proteomic studies. We have successfully constructed the recombinant adenovirus which encodes HCV NS4B. After adenoviral transduction NS4B protein expression was found to be moderately at 60 hours post transduction in Huh7 cells. Due to this we have opted the 60 hour

timepoint for our microarray studies. Microarray analysis showed that approximately 19 genes were differentially expressed upon NS4B expression. Pathway analysis of the microarray results showed that the majority of the genes were involved in the heteromeric pathway and the Wnt signalling pathway. The Wnt signalling pathway is mainly involved in the regualtion of cell adhesion, morphology, proliferation, cell migration, and structral remodelling. Activation of Wnt signals will leads to the accumulation of beta catenin levels in the cell and under normal contitions it is always associated with e-cadherins and cytoskeleton to maintain the cell shape. The upregulation of beta catenin levels will promote the nuclear accumulation and this will activate the transcription from the Tcf promoter.Transcription from the Tcf promoter will leads to cell proliferation and differentiation events. NS4B protein is reported to possess the enzymatic activity which can hydrolyze the ATP and GTP (Thompson *et al.*, 2009). It has also been reported that beta catenin, one of the key componenets of the Wnt signalling pathway, was associated with HCV induced heaptocellular carcinoma (Tien *et al.*, 2005). The core protein of HCV has been reported to activate the Wnt/beta catenin signalling cascades in SMMC-7721 cell line (Liu *et al.*, 2010). Even though NS4B poses the adenylate kinase activity, our array results have shown that NS4B induced activation of functionally similar genes of the host cell. These genes were responsible for the catalytic activity which leads to the synthesis of ATP and AMP from two ADP molecules. Therefore the upregulation of these genes by NS4B may lead to energy autonomy for the life cycle of the virus. Proteins like 2c protein of polio virus, is known to cause the membrane alterions depending on NTPase activity encoded by this protein (Bienz *et al.*, 1990; Rodriguez & Carrasco, 1993). Indeed cellular membrane rearrangements and intracellular vesicle trafficking were completely regulated by the NTPaes such as the small GTPases of the Rho family. Thus the NTPase activity of NS4B may also be linked with intracellular movements and membrane rearrangements (Ridley, 2006), which may be one of the reasons for the NS4B induced upregulation of the host genes resposnsible for the GTPase activity and hydrolase acitivty. Recent studies on the NTPase actitvites of NS4B protein of HCV have shown that it can transform the NIH3T3 cells without ras oncogene (Einav *et al.*, 2008). This clearly shows that NTPase actitvation of NS4B may be one of the causes for the hepatocellular carcinoma. Actiavtion of Wnt signalling pathway by NS4B in our array results also reflects its potential oncogenic activities. NS5A protein of HCV was shown to activate, interact and stabilize the beta catenin levels and also modulate the PI3 kinase pathway (Milward *et al.*, 2010; Park *et al.*, 2009a). Sterol regulatory element binding proteins (SREBP) are the major transcriptional factors in lipogenic gene expression were activated by NS4B protein through the PI3K pathway (Park *et al.*, 2009b). Our microarray

studies have also found the NS4B induced modulation of the PI3K pathway. Rab proteins like 5, 7 and 11 have been reported with formation of NS4B foci of membranous web and knockdown of Rab 5, 7, and 11 reduced the replication efficiency of HCV replication (Manna *et al.*, 2010). We also found upregaultion Rab proteins like RAB11b & Rab39b. Our data support the reports on other aspects of HCV replication which also shows that NS4B exploits the endocytic RAB proteins of host cells. The array results are in agreement with published studies. NS4B expression in Huh7 cells modulates different cellular pathways and molecular functions of the host cell. To better understand the biological significance of our array data, further studies has to be done by using the advanced research tools like HCVcc infectious system.

4.3. NS4B induced phopshorylation of cellular proteins

Phosphorylation of cellular proteins usually plays a major role in controlling cellular pathways. Most of the viruses are known to modulate the phopsho status of many key proteins which plays major role in the pathways. In order to screen NS4B induced modulation of the phopsho staus of host proteins, we have used the human phopsho kinase protein array. Stable expression of NS4B protein in Huh7 cells is known to activate SREBP by the phoshorylation of AKT at serine 473 (Park *et al.*, 2009b). However the Phospho proteomic screening of host cells upon NS4B expression have to date been largely unsuccessful. We have used the recombinant adenovirus expressing NS4B protein for these studies. Our phospho kinase array analysis of two independent experiments have shown the upregualtion of total beta catenin levels, and increased phopshorylation of GSK3 α + ß. In addition protein array results are in agreement with our microarray data with regard to the modulation of Wnt signalling pathway. The protein -array results were confirmed by Western blotting techniques. Fig. 22 also clearly showed the time dependent phopshoryaltion of GSK3 at 24 hours and 48 hpt of Ad-NS4B in Huh7 cells. Earlier studies on HCV NS5A protein also has shown an accumulation of beta catenin levels upon the phopshorylation by GSK3 (Milward *et al.*, 2010). Accumualtion beta catenin in response to Wnt signals will lead to the nuclear accumulation beta catenin in cells (Widelitz, 2005).

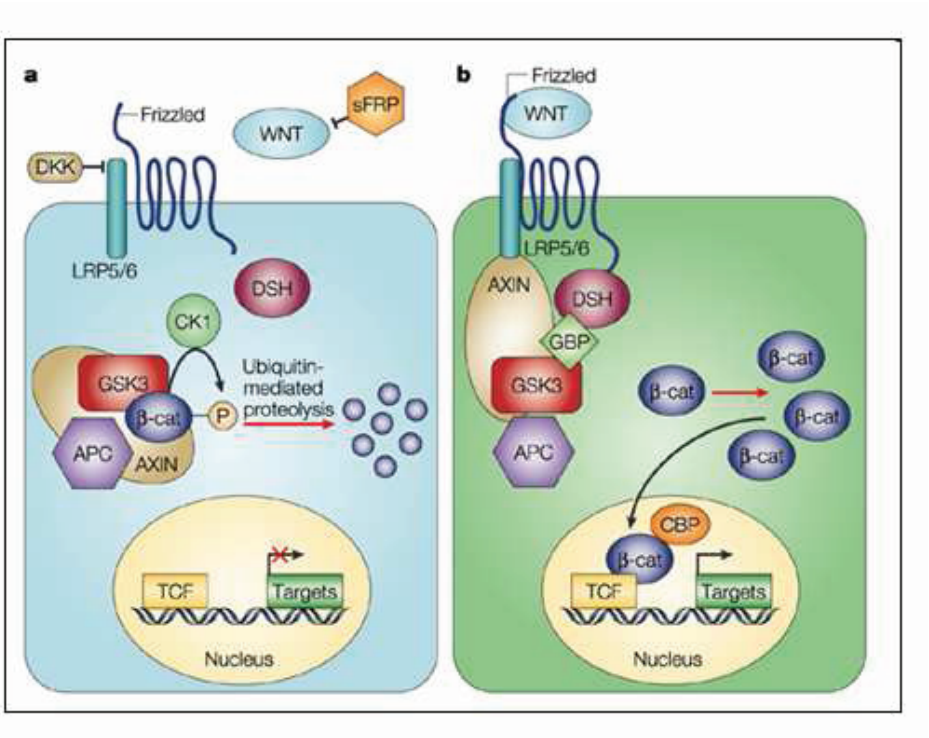

Figure 25: WNT/ ß-beta-catenin signaling mechanisms. (a) In the absence of Wnt signals beta cetnin is degraded by proteasome. (b) Activation of Wnt signaling promotes the nuclear entry of beta catenin and activates the target genes (Moon *et al.*, 2004). (Adapted with permission from Nature publishing group, license Number: 2686131401987).

NS5A of HCV has been shown to inactivate GSK3 beta by phopshoryaltion that in turn induces the stabilization of beta catenin and thus stimulates the transcription of beta catenin in PI3K dependent manner. It is also known that NS5A directly interacts with beta catenin (Milward *et al.*, ; 2010; Park *et al.*, 2009a; Street *et al.*, 2005). The accumulation of beta catenin leads to nuclear entry and pariticipation in the the formation of transcriptionally active complexes with members of the Tcf/Lef family (Barker *et al.*, 2000). So the next question in our studies was to check whether NS4B induced accumualtion of beta catenin leads to nuclear entry.For this purpose cells were fractionated into cytosol and nuclear fractions and analyzed by western blotting. We found an increase of nuclear beta catenin levels in NS4B expressing cells 90 hpt. Similar results were obtained in the nuclear extracts of HepG2 cels. In the absence of Wnt signals beta catenin is constantly degraded by protesome. The degradation of beta catenin is strictly regulated by the phopshoryaltion of beta catenin by GSK3 (Aberle *et al.*, 1997; Behrens *et al.*, 1998; Orford *et al.*, 1997). Therefore Wnt signalling is suggested to inhibit the phopshoryaltion of beta catenin and thus induce the accumulation of beta catenin. So the phopshoryaltion of beta catenin controls the protein elvels of beta catenin and thereby

the Wnt signaling pathway. We also checked the stability of beta catenin in the nuclear fraction based on the phopsho status of beta catenin at 90 hours and we found that it was not phopshorylated. This clearly shows the stability of nuclear localized beta catenin induced by HCV NS4B protein. Modulation of beta catenin levels were reported to be associated with HCV induced hepatocellular carcinoma (Huang *et al.*, 1999). Based on the microarray and phospho proteomics analysis we found that NS4B modulates the activation of Wnt signaling pathways in Huh7 cells. These results may be an additional evidence to explain oncogenic acitivites of NS4B protein and the molecular mechisms of HCV induced hepatocellular carcinoma.

Chapter 2

5. Hepatitis E virus

5.1. Introduction

Hepatitis E virus (HEV), the causative agent of viral hepatitis, is a non-enveloped positive-stranded RNA virus (Ahmad *et al.*, 2011; Chandra *et al.*, 2008). The first epidemic report for HEV was reported from Delhi, India in 1955 (Sidhu & Nair, 1957). This epidemic was caused by a flood in the river Yamuna in November 1955 leading to contamination of the city's water supplies. Almost all the reported epidemics occurred through the feco-oral route of transmission (Aggarwal, 2011). Symptoms associated with HEV infection are typically viral hepatitis and jaundice, malaise, anorexia, nausea, abdominal pain, fever and hepatomegaly (Smith, 2001). The mortality rate due to HEV infection was reported to be about 0.2%-1% among the general population. However the severity of the HEV infection is reported to be high in pregnant women with mortality rates of 15-20% (Bhatia *et al.*, 2008; Kar *et al.*, 2008; Pal *et al.*, 2005). HEV is grouped to the genus hepevirus, and family hepaviridae. Sequence analysis studies on HEV isolates form human and other mammals to the classification of HEV into different genotypes namely 1, 2, 3, and 4, and at least 24 subgenotypes (1a-1e, 2a-2b, 3a-3j and 4a-4g) (Lu *et al.*, 2006). Based on the outbreak reports hepatitis E is highly endemic in Indian subcontinent, China, South east and Central Asia, the Middle East, and northern and western parts of Africa (Corwin *et al.*, 1999). Fig. 26 shows the geographical distribution of highly endemics regions for HEV.

5.1.1. Genome organization of HEV

HEV is a non-enveloped positive-stranded RNA virus with an icosahedral capsid of about 27 to 34 nm. The single stranded RNA genome is 7.2 kb long, polyadenylated with short non coding regions at each end and three partially overlapping open reading frames (ORF), called ORF1, ORF2, and ORF3 (Ahmad *et al.*, 2011; Tam *et al.*, 1991). The 186 kDa ORF1 protein of HEV contains several conserved motifs which encode methyltransferase, papain-like cysteine protease (PCP), RNA helicase and RNA dependent RNA polymerase (RdRp) (Agrawal *et al.*, 2001; Ropp *et al.*, 2000). Earlier reports suggested HEV to have a capped RNA genome due to the methyl transferase motiff (Magden *et al.*, 2001). ORF2 of HEV represents the capsid protein of 660 amino acids which encapsidate the viral RNA genome. This capsid protein is known to be glycosylated at Asn 310 position and is stabilized under acidic pH (Graff *et al.*, 2008; Surjit *et al.*, 2007; Zafrullah *et al.*, 1999). ORF3 protein encodes

a protein of 13.5 kDa which contains a P1 region known to be phosphorylated at serine 80 (Korkaya *et al.*, 2001). This protein has also been shown to modulate host cellular pathways and prevent the cell death (Moin *et al.*, 2007).

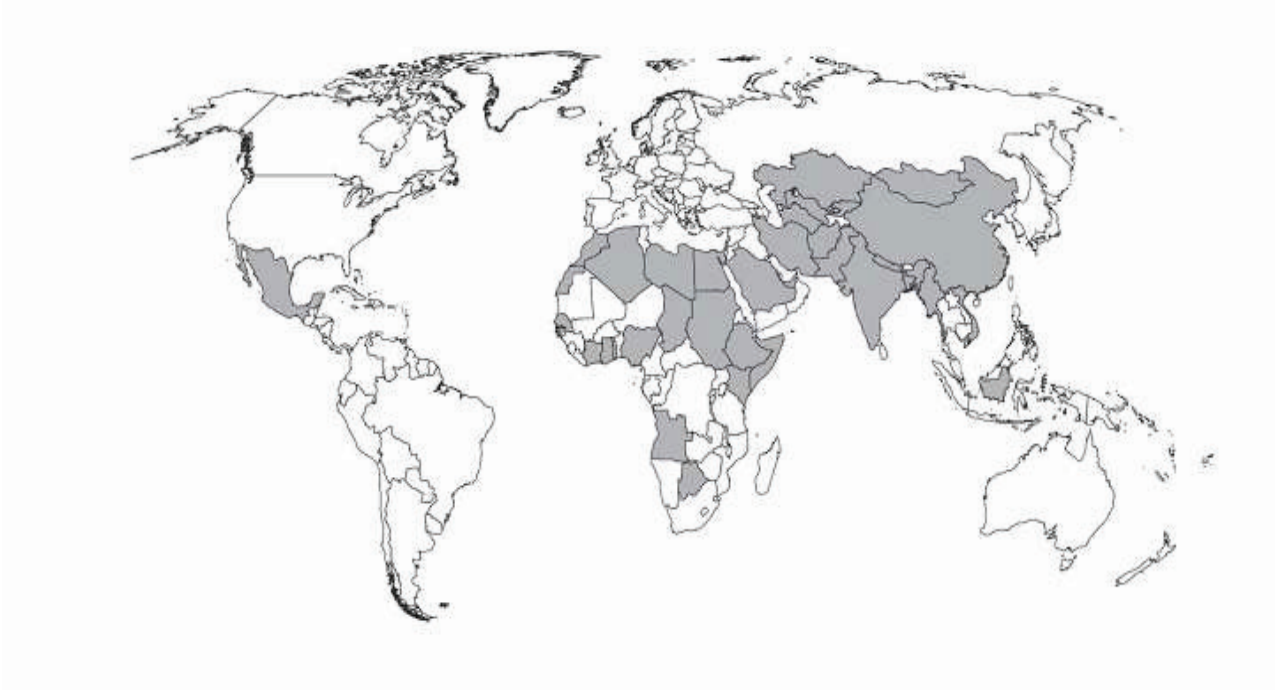

Figure 26: Geographical distribution of HEV endemic areas. Grey colour represents the highly endemic regions of HEV infection (Adapted with permission from John Wiley and Sons publishing group, license Number: 2675270517322**)**

5.1.2. General features and cellular interactions of HEV ORF2 protein

The viral capsid protein of HEV is encoded by ORF2. The ORF2 segment of the HEV genome comprises 1980 nucleotides. This gene encodes the main structural polypeptide of HEV. Sequence analysis has shown that ORF2 encodes a large hydrophobic domain at the N-terminus and this region has a typical signal sequence and contains a potential cleavage site (PA/PPP) (Tam *et al.*, 1991). This region is mainly concerned with virion maturation and also the ER translocation signals, while the C-terminal region of the protein encodes an RNA binding site (Surjit *et al.*, 2007). Heterologous expression analysis shows that ORF2 protein accumulates in the ER initially and a fraction of this protein will be translocated back to the cytosol. However HEV ORF2 protein remains in the cytosol without being a substrate of the 26S proteasome. Processing of ORF2 protein in the ER leads to ER stress and activates the ER chaperones. The glucose-regulated chaperone proteins 78 kDa (GRP78) and 94 kDa (GRP94)

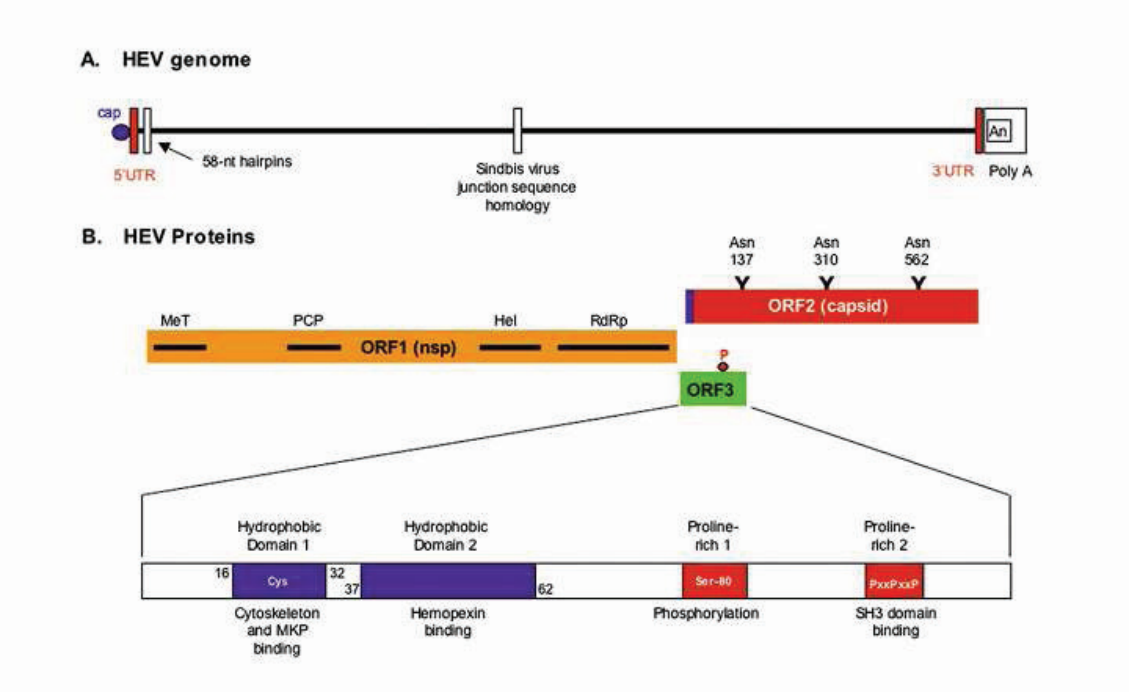

Figure 27: Genome organization and proteins of HEV. (A) The ~7.2 kb positive strand RNA genome is capped at the 5′ end and the 3′ end is polyadenylated. The untranslated regions (UTR) are located at both ends colored as RED. (B) Nonstructral proteins (nsp) were encoded by the ORF1 that codes for various functional units–methyltransferase (MeT), papain-like cysteine protease (PCP), RNA helicase (Hel) and RNA dependent RNA polymerase (RdRp). The viral ORF2 encodes the viral capsid protein; the blue colour represents the N-terminal signal sequence and glycosylation sites. The small phopshoprotein encoded by ORF2 and its known domains were represented as blue and red (Chandra *et al.*, 2008). (Adapted with permission from Springer publishing group, license Number: 2675351252982)

have been found to be up-regulated upon ORF2 expression (Surjit *et al.*, 2007; Zafrullah *et al.*, 1999). The interactions of the capsid protein of HEV with host factors was studied using the truncated viral structural peptide, p239 (ORF2, aa368-606). Based on proteomics and immunofluorescence studies HSP90 was found to be aasociated with p239 in a complex. This ORF2/p239 complex as a transporter for HEV capsids to the cytoplasmic destination (Zheng *et al.*, 2010). In addition this protein is also known to interact with GRP78 an ER chaperone (Yu *et al.*, 2011). Overload in ER with misfolded proteins will cause ER stress and this will lead to the activation of UPR pathways. ORF2 protein is known to cause ER stress, which also activates the ER chaperones like GRP78 and GRP94. Up regulation of these chaperones is known to be associated with the unfolded protein response which binds to and retains the

misfolded protein in the ER. ER stress response in mammalian cells involves the major two phases of adaptation and apoptosis. During the adaptation stage cells will use the ER chaperones like GRP78 and GRP94 to refold the unfolded proteins to maintain the ER homeostasis (Little *et al.*, 1994; Ni & Lee, 2007). However if this adaptation process fails, then the pro-apoptotic process will be initiated by the ATF6 and ATF4 dependent transcriptional activation of C/EBP homologous protein CHOP. The response elements of the CHOP promoter are well characterized by promoter mapping studies and have been defined as follows: AARE2 (bases -778 to -770), AARE1 (bases -310 to -302), AP1 element (bases –244 to -238), and two ERSE (bases -103 to -76)(Guyton *et al.*, 1996; Kwok & Daskal, 2008; Ubeda & Habener, 2000; Yamazaki *et al.*, 2010). In mammalian cells ER stress response consists of three different pathways that are mediated by PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6a (ATF6a and ATF6b) and Ire1b and Ire1b (Ma *et al.*, 2002). The capsid protein of HEV contains N-linked oligosaccharides and exist as glycosylated and non glycosylated forms (Torresi *et al.*, 1999; Zafrullah *et al.*, 1999). Mutational analysis of these potential glycosylation sites prevents the formation infectious viral particles (Graff *et al.*, 2008). The C-terminal region of ORF2 contains several antigenic sites including a neutralization epitope ranging from residues 452 to 617 (Aggarwal *et al.*, 2007). Previous studies investigating humoral responses against HEV showed prominent antibody responses against the antigenic epitopes of ORF2 (Tsarev *et al.*, 1997). Thus, ORF2 and ORF2 derived peptides have been used as vaccine candidates (Shrestha *et al.*, 2007; Zhu *et al.*). However, very little is known about the host cellular targets of ORF2 protein.

5.2. Materials and Methods

5.2.1. Materials

Materials and devices if not mentioned here are described in chapter 1.

5.2.1.1. Devices

Confocal microscope	Leica Confocal Microscope, Wetzlar,
Fluorescence associated cell sorter (FACS)	BD Biosciences, New Jersey, U.S

5.2.1.2. General material

Coverslips 12 mm (round), 18 mm x 18 mm (square)	Gerhard Menzel GmbH, Braunschweig
Microscope slides (26 mm x 76 mm x 1 mm)	Engelbrecht, Edermünde

5.2.1.3. Chemicals

DTT	Sigma Aldrich, St. Louis, USA
Thapsigargin	TOCRIS, Missouri, USA
Mounting medium	Dako, Carpinteria, USA

5.2.1.4. Primary Antibodies

Anti- Hsp72 (mouse)	Stressgen Biotechnologies
Anti- His probe (Rabbit)	Sigma Aldrich, St. Louis, USA
Anti- Bax N-20 (Rabbit)	Sigma Aldrich, St. Louis, USA
Anti- eIF2α (Rabbit)	Cell Signaling Technology, Danvers, USA
Anti- phospho-eIF2α/Ser51 (Rabbit)	Cell Signaling Technology, Danvers, USA
Anti- COX IV (Rabbit)	Cell Signaling Technology, Danvers, USA

5.2.1.5. Secondary Antibodies

Goat anti-mouse IgG Alexa Fluor 633 conjugate	Molecular Probes, via Invitrogen, Karlsruhe

5.2.1.6. Vectors

pGL3-CHOP luciferase promoter, deletion	Kind gift of Pierre Fafournoux INRA, France

ATF4 (-318 to -286), ERSE (-221 to -40)	
pGL3-AARE1, pGL3-AARE2, pGL3-ERSE pGL3-AP1	Kind gift of S.C.M. Kwok. Albert Einstein Medical center, U.S.A
pcDNA3.1-HEV ORF2	Dr. Saijo Thomas, VEGT, Rostock
pcDNA3.1-Chikungunya virus capsid protein	Dr. Saijo Thomas, VEGT, Rostock

5.2.1.7.Adenovirus

Ad-ORF2	Dr. Saijo Thomas, VEGT, Rostock
Ad-GFP	Anja Stoll, VEGT, Rostock
Ad-p53	Anja Stoll, VEGT, Rostock

5.2.2. Expression analysis of HEV ORF2 in Huh7 cells

5.2.2.1. Transfection

Expression of HEV ORF2 protein in Huh7 cells was tested by western blotting. The Huh7 cells were seeded in 10 cm cell culture dishes 24 h before transfection. After 24 hours the cells were transfected with 4µg of pcDNA3.1 HEV ORF2 using the transfection reagent effectene according to the instructions of the manufacturer.

5.2.2.2. Western blotting

After 48 hours post transfection the protein lysates were prepared, and western blotting was performed as mentioned in section 2.8.2. An anti-His probe was used to detect the hist tagged ORF2 protein

5.2.3.Promoter activation assays of the pro-apoptotic gene CHOP

5.2.3.1. Cell culture and transfection

Huh7 cells and H1299 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. For transfection, Huh7 and H1299 cells were seeded in 6-well plates at a density of $2x10^5$ cells and transfection was performed using effectene (Qiagen) transfection reagent according to the manufacturer's instruction. Huh7 and H1299 cells were transfected with 0.1µg of pGL3CHOP promoter luciferase reporter plasmid and co-transfected with pcDNA3.1-HEV ORF2 effector plasmid DNA (0.25 µg, 0.5 µg, 1.0 µg). The AARE1, AARE2, ERSE, AP1 enhancer luciferase reporter constructs of the CHOP promoter 0.1 µg each was similarly co-

transfected in Huh7 cells along with (1.0 µg) pcDNA3.1-HEV ORF2 effector plasmid. Huh7 cells were transfected with (0.1 µg) of deletion constructs delta ATF4 and delta ERSE of CHOP promoter constructs with 1.0 µg of pcDNA3.1-HEV-ORF2 effector plasmid.

5.2.3.2. Luciferase reporter assay

Cells were harvested 48 hours after transfection. Briefly, 150 µl of cell lysis buffer was added to the cell pellet and incubated on ice for 1 hour after vortexing. After the incubation the cell extracts were centrifuged at 12,000g for 2 minutes and the supernatant was used for the luciferase activity assay. Luciferase activity readings were taken on a luminometer. Data were normalized to the total protein levels and are expressed as fold activation over pcDNA 3.1 alone (which was given an arbitrary value of 1).

5.2.3.3. Quantitative real time PCR analysis of CHOP gene expression

Huh-7cells were transduced with an MOI of 20 with either adenovirus expressing HEV ORF2 (Ad-ORF2) or by using an adenovirus expressing green fluorescent protein (Ad-GFP), which served as a negative control. Total RNA was isolated 48 hours hpt using the RNeasy Mini Kit (Qiagen). A total of 1 µg RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-dT. cDNA samples were mixed with Qiagen Quantitect Master Mix and analyzed on BIORAD iQ5 Multicolor Real-Time PCR Detection System using the following primers for CHOP, fwd 5’ AGCTGGAACCTGAGG 3’ rev 5’ TGGATCAGTCTGGAA 3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run as an endogenous control and all samples were normalized to the GAPDH expression levels

5.2.4. Microarray analysis of Huh7 cells upon HEV ORF2 expression

5.2.4.1. Microarray analysis

Huh-7cells were transduced with an MOI of 20 with either Ad-ORF2or by using Ad-GFP as a negative control. Total RNA was isolated 60 hpt using the RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was used to make biotinylated cRNA probes which were hybridized to Affymetrix Human Genome U133 Plus 2.0 Array according to the supplier’s instructions (Affymetrix). Microarrays were analyzed by laser scanning (Affymetrix GeneChip Scanner 3000). Three independent experiments, with six chips per independent experiment (incorporating a dye swap), were undertaken and the data were analyzed using the MAS5

software (Microarray suite, Affymetrix). The fold changes were calculated as log 2 of signal log ratio and the cut-offs were set at 1.7 fold and a p-value cut-off at 0.05.

5.2.4.2. Validation of microarray results by qRT-PCR analysis

RT-PCR was performed on total RNA prepared by Nucleospin RNAII (Macherey–Nagel). A total of 1µg RNA was reverse transcribed using Omniscript RT (Qiagen) and Oligo-dT. cDNA samples were mixed with Qiagen Quantitect Master Mix and analyzed on BIORAD iQ5 Multicolor Real-Time PCR Detection System using the following primers. Primers for Hsp72 fwd 5'-ACCTTCGACGTGTCCATCCTGA-3', rev 5'-TCCTCCACGAAGTGGTTCACCA-3', Hsp70B' fwd 5'-CCCTAAGGCTTTCCTCTTGC-3', rev 5' -CATGAAGCCGAGCAGTACAA-3; Expression levels of Hsp40/DNAJ4 were detected by using the specific Taqman probe Hs00388055-m1α. GAPDH) was used as an endogenous control and all samples were normalized to the GAPDH expression levels.

5.2.5. Nuclear accumulation of HSP72

5.2.5.1. Nuclear fractionation

The cytoplasmic and nuclear fractions of protein samples were prepared 72 hpt using the Nuclear Extract kit (Active Motif). The cytoplasmic fractions were harvested as supernatants and the pellets were resuspended in 50 µl of complete lysis buffer and centrifuged at 14,000g for 10 min at 4°C. The nuclear fraction was collected as supernatant. Both the protein fractions were immunoblotted and probed with appropriate primary antibodies Hsp72, TBP and β-Actin. Signal intensities of Hsp72 for both nuclei and cytoplasm were quantified and normalized to the appropriate loading controls and a nuclear cytoplasmic ratio of Hsp72 was calculated as described previously.

5.2.5.2. Hsp72 immunofluorescence

Huh7 cells grown on coverslips were transduced with either Ad-GFP or Ad-ORF2. After 72 h cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. The coverslips were blocked with 5% BSA in PBS for 1 h at 37 °C and incubated with Hsp72 antibody (1:50 dilution), as described elsewhere. A secondary antibody conjugated to Alexa Fluor 633 was used for the visualization with a laser-scanning microscope.

5.2.6. Interaction of HSP7 and ORF2

5.2.6.1. Co-immunoprecipitation

A total of 200 µg of protein lysate from the HEK293 cells transfected with pcDNA3.1-HEV ORF2 were precipitated with 2 µg of Hsp72 antibody. Protein A/G agarose beads (Santa Cruz) were used to collect the immunoprecipitated complexes and the beads were washed with PBST before performing SDS PAGE and Western blot analysis.

5.2.6.2. Molecular docking analysis

The structure of the Hsp72 (PDB ID LYUW) and the HEV ORF2 (PDB ID 2zzq_A) protein was modelled using automated homology modeling server 3D-JIGSAW. Both the PDB structures were submitted to Patchdock server with Hsp72 to act as a receptor and ORF2 as a ligand with the default parameters. The top 1000 conformations were further refined using the Firedock server. Complexes with lowest global energy were selected and further analyzed. The figures were generated using pymol (http://www.pymol.org).

5.2.7. Analysis of apoptosis upon ORF2 expression

5.2.7.1. Flow cytometry

Huh7 and HepG2, cells grown on 10 cm cell culture dishes were transduced with either Ad-GFP or Ad-ORF2. An MOI of 20 was used for transduction. After 72 hours, cells were harvested, fixed in 70 % ethanol and stained for DNA content with propidium iodide as described elsewhere. Analysis was done in a FACS Calibur flow cytometer (Becton Dickinson) using Cell Quest Software.

5.2.7.2. Cellular fractionation

For sub-cellular fractionation, the Apo Alert Cell Fractionation Kit (Clontech) was used according to the manufacturer's recommendations. Briefly, after 72 hpt, cells were harvested, washed twice with PBS, resuspended in cell fractionation buffer and homogenized. Cytosolic and mitochondrial extracts were fractionated by differential centrifugation. Protein samples (50 µg) from both fractions were separated on 12% SDS-PAGE and immunoblotted with COX IV and Bax N-20 antibodies.

5.3. Results

5.3.1. ORF2 activates the CHOP promoter

Processing of HEV viral proteins leads to the accumulation of ORF2 in the ER. Ultimately, this accumulation causes ER stress. Up-regulation of the ER resident chaperones GRP78 and GRP94 as well as the protein disulfide isomerase has been shown to be up-regulated in ORF2 expressing cells (Surjit *et al.*, 2007). A failure of this ER stress adaptation system and overexpression of the ER chaperones like GRP78 would also lead to the activation of pro-apoptotic downstream target genes like CHOP (Ma *et al.*, 2002). Since ORF2 expression has been shown to induce ER stress and activation of ER chaperones, we analyzed whether expression of ORF2 had any effect on the expression of the pro-apoptotic gene CHOP. Using a transient transfection system we looked at the transcriptional activation of CHOP in cells expressing the ORF2 protein. We have used a luciferase reporter construct driven by the full-length 954bp CHOP promoter and either pcDNA3.1 or pcDNA-HEV ORF2 were transiently transfected into Huh7 cells. We found that expression of ORF2 caused activation of the CHOP promoter in a dose-dependent manner (Fig. 28a). Similar findings were also observed in the non-hepatic cell line H1299 (Fig. 28b). As a control for the specificity of CHOP activation by HEV ORF2 we used the capsid protein of Chikungunya virus and we found that it had no effect on the CHOP promoter activity (Fig. 29)

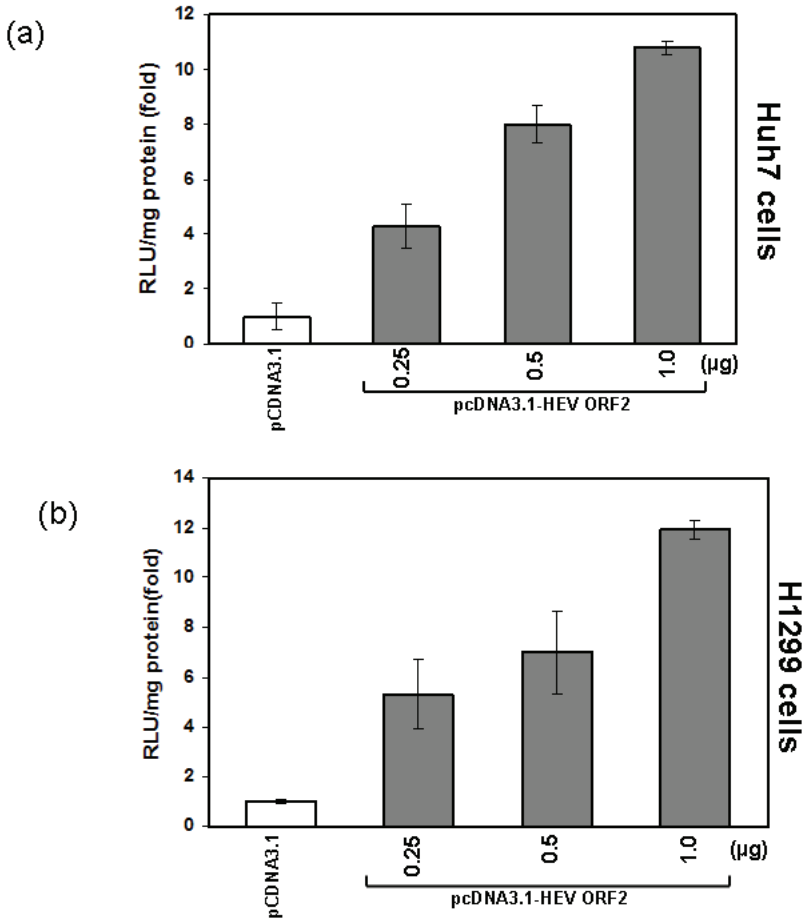

Figure 28: ORF2 activates transcription from full length CHOP promoter. Huh7 (a) H1299 cells (b).

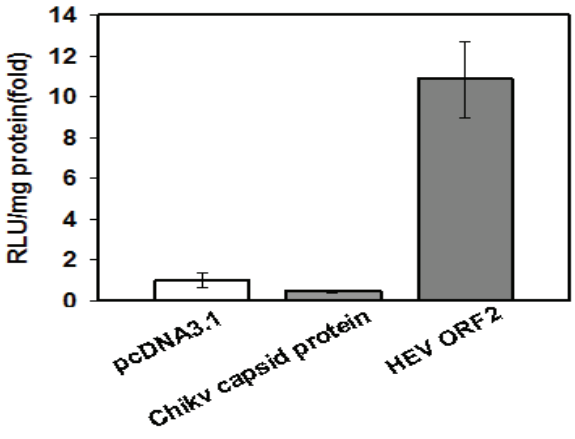

Figure 29: Promoter activation assay of of CHOP after 48 hours post transfection

The specificity of the CHOP activation by ORF2 was also confirmed by the inability of ORF2 to activate a non-UPR gene promoter (data not shown). We also observed an increase of the

CHOP mRNA levels in ORF2 expressing cells and thapsigargin treated cells which served as a positive control (Fig. 30).

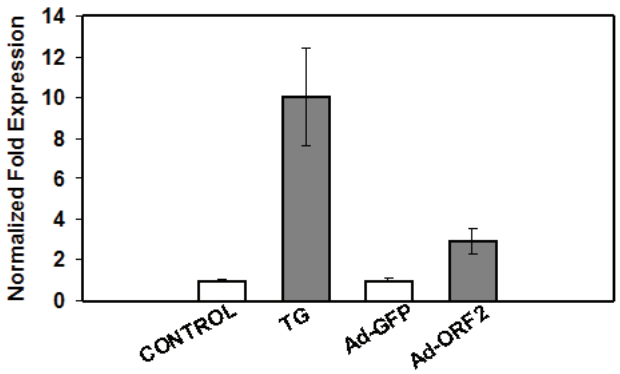

Figure 30: Quantitative PCR analysis of CHOP mRNA levels after 48 hpt with Ad-GFP or Ad-ORF2. Thapsgargin (4μM) treated cells for 4 hours were used as a positive control

5.3.2. ORF2 activates both, AARE and ERSE elements of the CHOP promoter

The CHOP gene expression is regulated mainly through the regulatory sites ERSE, AARE1, and AARE2, respectively. In response to oxidative stress CHOP gene expression can also be activated through the AP1 element (Oyadomari & Mori, 2004). To investigate the relative contribution of these elements in response to HEV ORF2 protein expression, we employed different constructs with the isolated response elements (AARE1, AARE2, ERSE, AP1) of the CHOP promoter fused with the luciferase reporter. While AARE2 and to a lesser extent AARE1 showed a strong activation by HEV ORF2, the ERSE and AP1elements of the CHOP promoter were only weakly activated by the capsid protein (Fig. 31a). We also tested the effect of promoter constructs with deletions encompassing the ERSE and activating transcription factor 4 (ATF4) binding sites of the CHOP promoter. Activation by HEV ORF2 was reduced upon the deletion of the ATF4 region as compared to the construct with deleted ERSE region of the CHOP promoter (Fig. 31b).

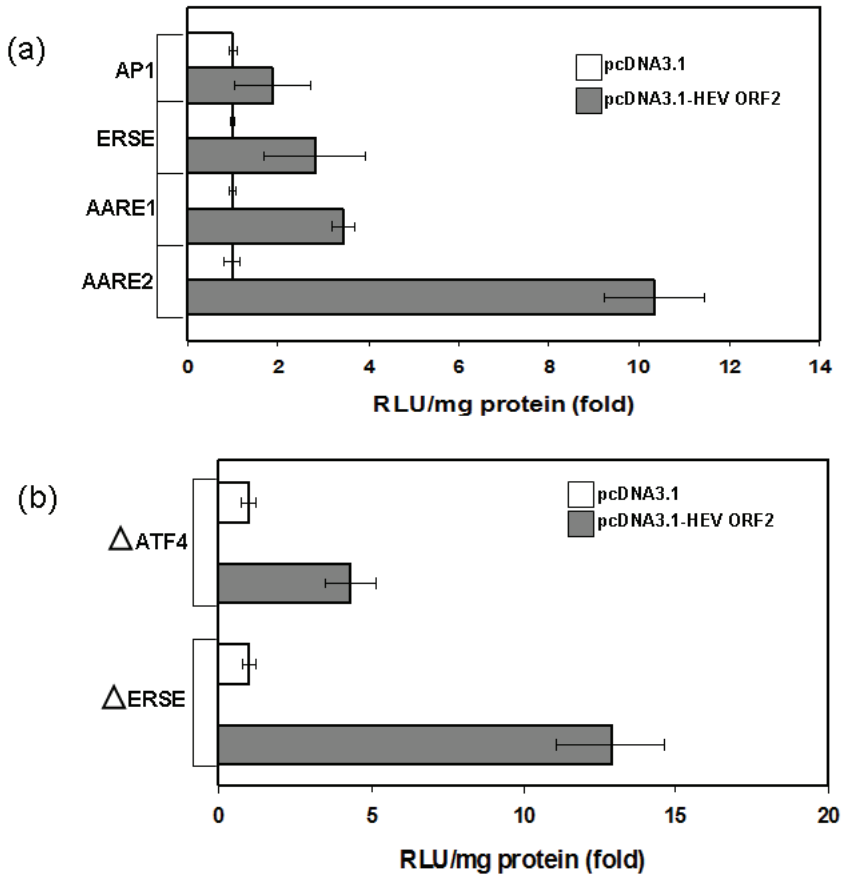

Figure 31: (a) Promoter activation assay of AARE1, AARE2, ERSE, AP1 regulatory sites of CHOP promoter 48 hours post transfection (b) Promoter activation assay of delta ATF4 and delta ERSE of CHOP promoter upon ORF2 expression.

These results suggest that the ATF4 binding sites contribute to the major part of the CHOP promoter activation by the ORF2 protein.

5.3.3. ORF2 activates the phosphorylation of eIF2α

It is well established that ER stress leads to the activation of the three signaling branches of UPR (Lin *et al.*, 2007). Our results showed that ORF2 induced activation of the CHOP promoter was mediated mainly through the AARE regulatory sites. Transactivation of the AARE regulatory sites of the CHOP promoter is dependent on PERK mediated eIF2α phosphorylation and ATF4 translation (Deshaies, 1999; Harding *et al.*, 2000; Ma *et al.*, 2002). Thus, we analyzed the phosphorylation status of eIF2α upon ORF2 expression. Fig. 32 a & b shows that in cells transduced with Ad-ORF2 increased phosphorylation levels of eIF2α without a concomitant increase in the total eIF2α levels.

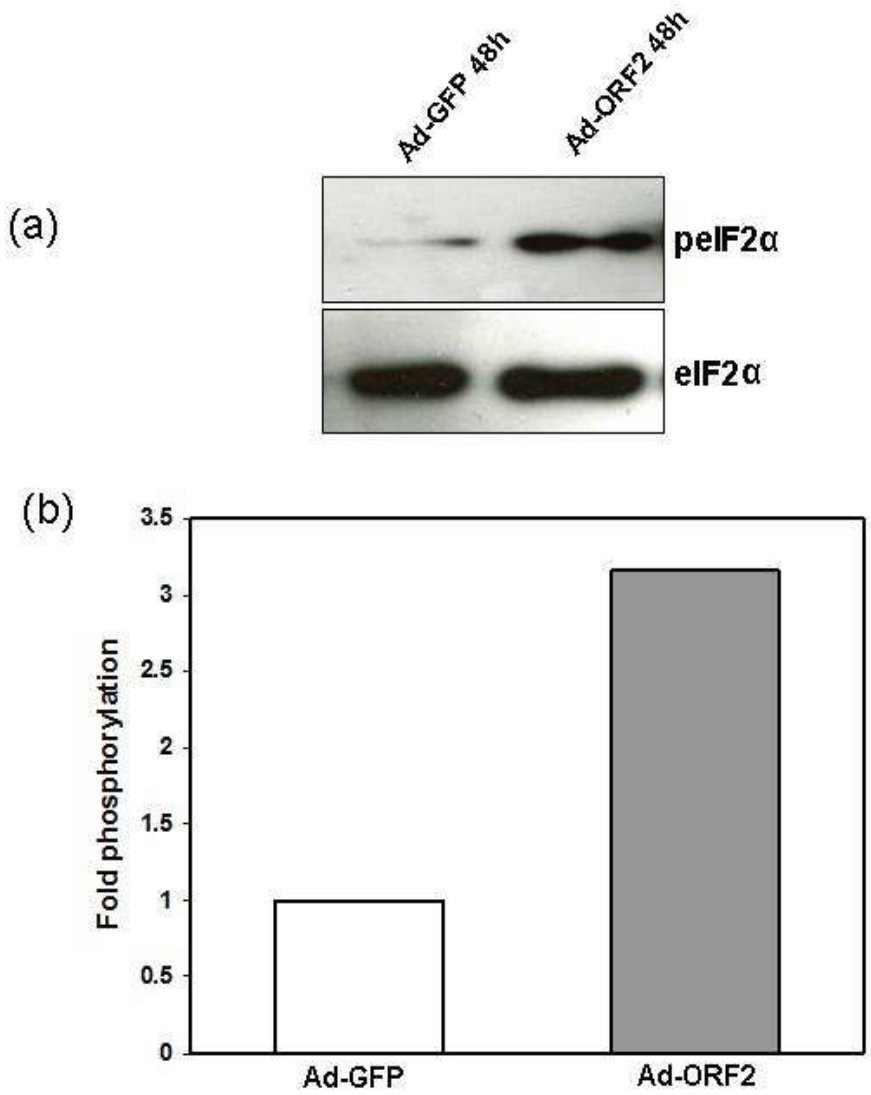

Figure 32: ORF2 activates PERK mediated eIF2α phosphorylation during ER stress. (a) Huh7 cells were transduced with either Ad-GFP or Ad-ORF2 for 48hours, uninfected cells were treated with thapsigargin (2μM) for 4 hours prior to harvest. Thapsigargin as a positive control for ER stress and cell lysates were separated by SDS PAGE and Western blotted for the indicated proteins. (b) Signal intensities from Fig. 32a were quantified, and phospho eIF2α signals were normalized to total eIF2αalpha levels.

These results suggest a possible mechanism for the transcriptional activation of AARE regulatory sites of the CHOP promoter where expression of ORF2 induces the phosphorylation of eIF2α.

5.3.4. Microarray analysis shows that the ORF2 induces the up regulation of Hsp70B', Hsp72 and Hsp40

The CHOP protein works as a transcription factor and can differentially regulate the genes involved in either survival or death (Zinszner *et al.*, 1998). Overexpression of CHOP is known to regulate the protein levels of anti-apoptotic Bcl2 family proteins and the translocation of the pro-apoptotic protein Bax from the cytosol to mitochondria (Matsumoto *et al.*, 1996; McCullough *et al.*, 2001). Besides induction of CHOP, ORF2 may induce additional signaling pathways counteracting pro-apoptotic signals of CHOP. To investigate the consequences of ORF2 induced CHOP expression, we investigated the overall modulation of transcriptional changes induced by the expression of ORF2 protein. Microarray analysis of the Huh7 cells transduced either with Ad-ORF2 or an Ad-GFP showed that expression of ORF2 induced a specific subset of chaperones like Hsp70B', Hsp72 as well as the co-chaperone Hsp40 (table.9).

Table 9. **Chaperones and co-chaperones regulated by ORF2.** The expression profiles of host genes significantly regulated (≥1.7 fold ; *p* value ≤ 0.05) at 60 hours post transduction of Huh7 cells with Ad-ORF2 versus Ad-GFP using Affymetrix HGU_133 plus 2.0 array analysis. All values are results of three independent experiments and the fold changes were calculated as log 2 of signal log ratio using MAS5 (Microarray suite, Affymetrix).

Affymetrix ID	Gene symbol	Description	Fold change
202581_at	Hsp72(HSPA1A)	Heat shock 70kDa protein 1A	1.7
200800_s_at	Hsp72(HSPA1A)	-	1.9
1554334_a_at	Hsp40(DNAJ4)	DnaJ (Hsp40) homolog, subfamily A, member 4	2.2
117_at	Hsp70B'(HSPA6)	Heat shock 70kDa protein 6 (Hsp70B')	3.5
213418_at	Hsp70B'(HSPA6)	-	7.6

qRT-PCR experiments confirmed the results of microarray analysis. ORF2 expression resulted in approximately 4-fold, 3-fold, and 20-fold for Hsp40, Hsp72, and Hsp70B', respectively (Fig. 33 a&b). The heat shock family of proteins prevents the irreversible aggregation of unfolded proteins and keeps them competent for refolding. The microarray and q-PCR results suggest that the expression of ORF2 protein leads to the coordinated regulation of chaperones as well as the co-chaperone in response to protein damaging stress with an increased burden of non-native protein conformations.

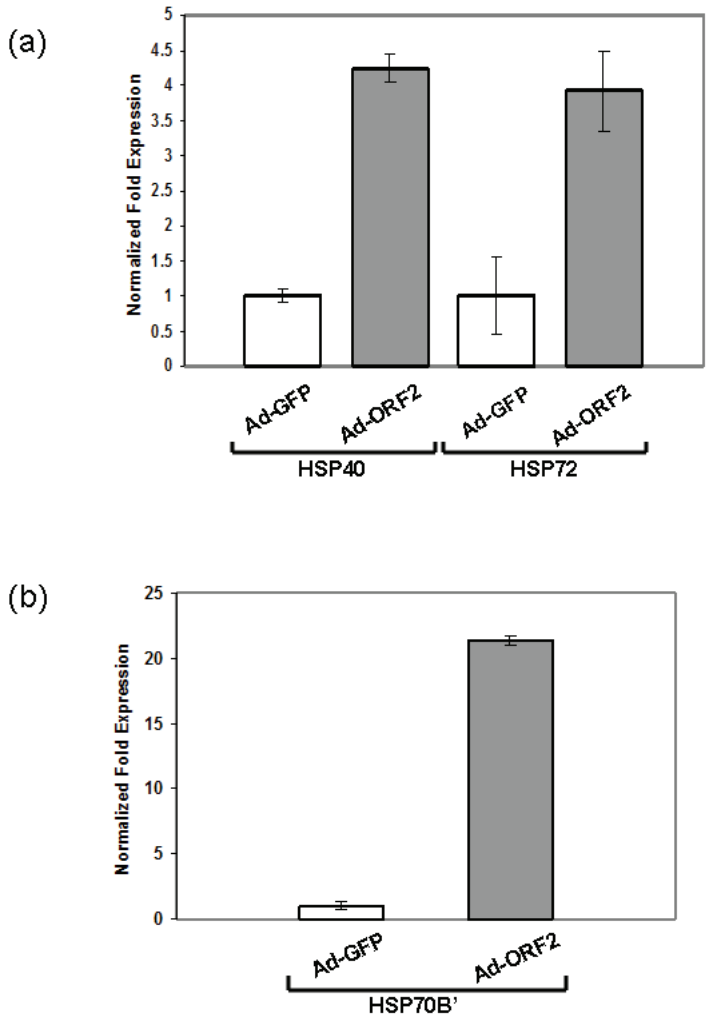

Figure 33: ORF2 up-regulates Hsp70B', Hsp72, and Hsp40. Quantitative RT-PCR analysis of heat shock proteins in Ad-ORF2 infected Huh7 cells. Expression levels were normalized with Ad-GFP transduced cells as control. Error bars indicate mean ± S.D. of three independent experiments.

5.3.5. ORF2 interacts with Hsp72

HEV ORF2 protein interacts directly with the members of the heat shock family of proteins GRP78, and Hsp90 (Yu *et al.*, 2011; Zheng *et al.*, 2010). Since we observed increased expression of Hsp72 we also looked at a possible direct interaction of ORF2 with Hsp72. Experiments were performed to determine whether ORF2 and Hsp72 interact in ORF2 expressing cells. Protein extracts from transfected cells were subjected to Co-IP with an antibody against Hsp72 or control IgG, and subsequently analyzed through Western blotting with an anti-His-probe polyclonal antibody which detects the His-tagged ORF2 protein.

Representative results demonstrate that HEV ORF2 co-precipitated with Hsp72 and not with the control antibody (Fig. 34).

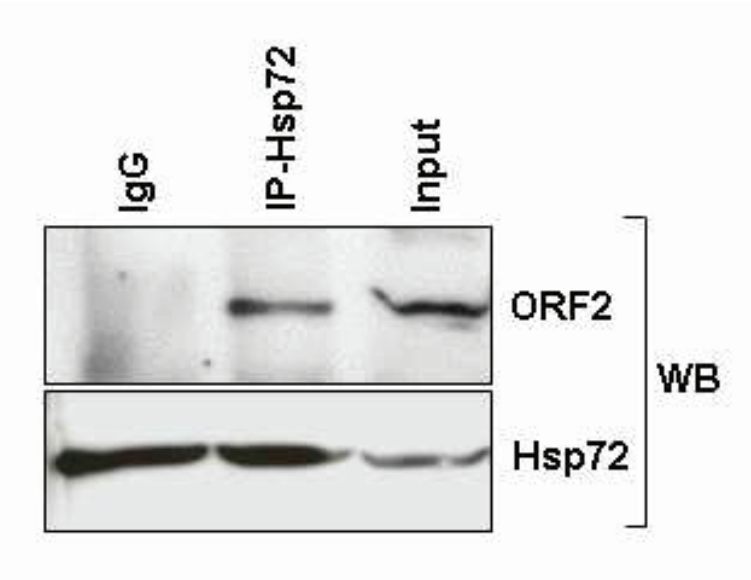

Figure 34: ORF2 interacts directly with Hsp72. HEK293 cells were transfected with pcDNA3.1-HEV ORF2 and the cell lysates were subjected to immunoprecipitation using an anti Hsp72 monoclonal antibody or control IgG. The immunoprecipiatates were subjected to immunoblot analysis to detect the His tagged ORF2 protein

5.3.6. *In silico* modeling of the ORF2 and Hsp72 interaction

We also have used the in silico based docking analysis program to analyze a probable interaction between Hsp72 and ORF2 protein (Fig. 35). The best fit model scores a global energy (GE) value of -71.90, attractive and repulsive van der Waals force (Avwf) of -45.23, repulsive van der Waals energy (Rvdw), 21.10 and atomic contact energy (ACE)-17.66. These data give additional evidence for the ORF2 –Hsp72 interaction. In addition to *in silico* modeling suggests that the C-terminal region of the ORF2 protein is indeed responsible for the interaction with Hsp72.

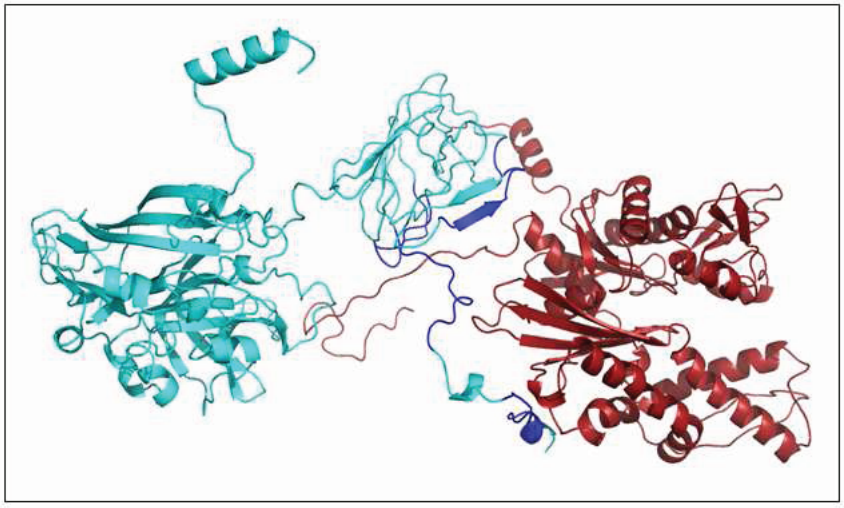

Figure 35: *In silico* modelling of ORF2 protein interactions with Hsp72. ORF2 and Hsp72 are displayed as a solid ribbon diagram, ORF2 (cyan), Hsp72 (firebrick red) and the interacting regions (blue).

5.3.7. ORF2 induces the nuclear accumulation of Hsp72

The major heat shock protein Hsp72 is well known for its critical role in cell survival and its strong anti-apoptotic effects by modulating several pathways involved in apoptosis (Chow *et al.*, 2009; Welch & Feramisco, 1984). As a part of its protective function, Hsp72 will migrate to the nucleus to execute extra chaperone activity in this compartment (Hageman *et al.*, 2007; Knowlton *et al.*, 2000). To investigate ORF2 associated nuclear translocation of Hsp72, we checked for the nuclear accumulation in ORF2 expressing cells by immunofluorescence and cellular fractionation methods. Huh7 cells transduced with Ad-ORF2 showed that ORF2 protein promoted nuclear accumulation of Hsp72 (Fig. 36).

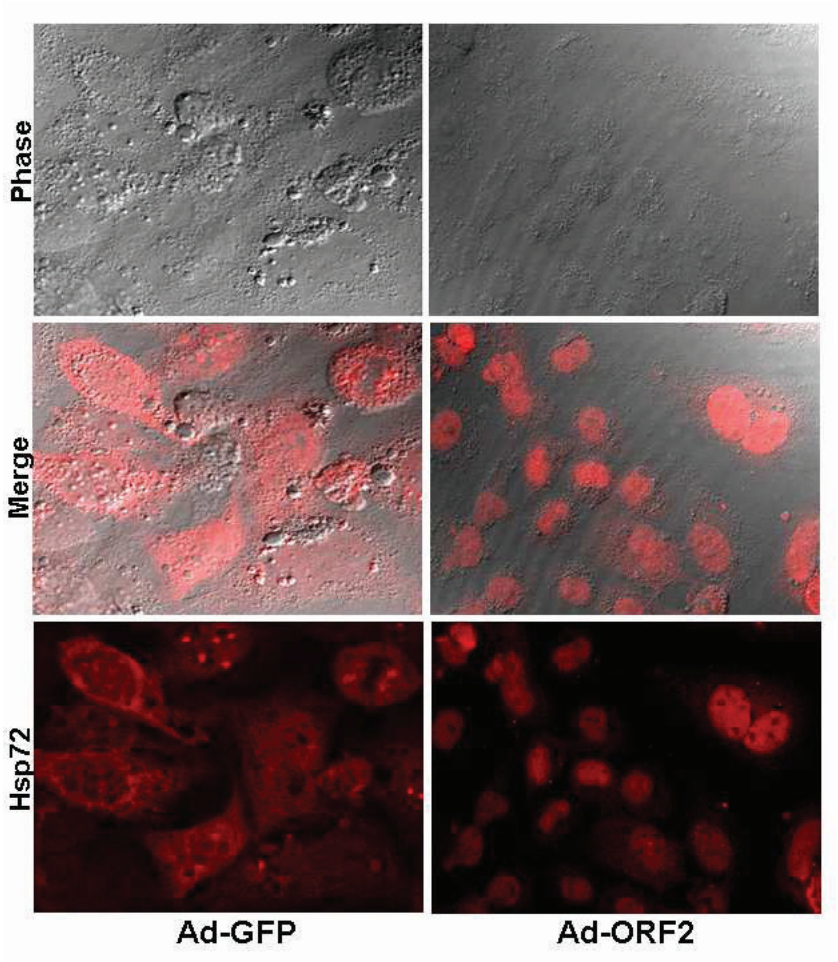

Figure 36: ORF2 increases nuclear accumulation of Hsp72. Huh7 cells were grown on cover slips placed in the 6 well dishes and transduced with either Ad-GFP or Ad-ORF2. After 72 hpt, the cells were fixed and stained with anti Hsp72 antibody as described in materials and methods. The cells were then imaged with a confocal microscope and the composite images were created using the IMAGE J software.

Nuclear accumulation of Hsp72 was mainly detected in the nuclear fraction of ORF2 expressing cells (Fig. 37).

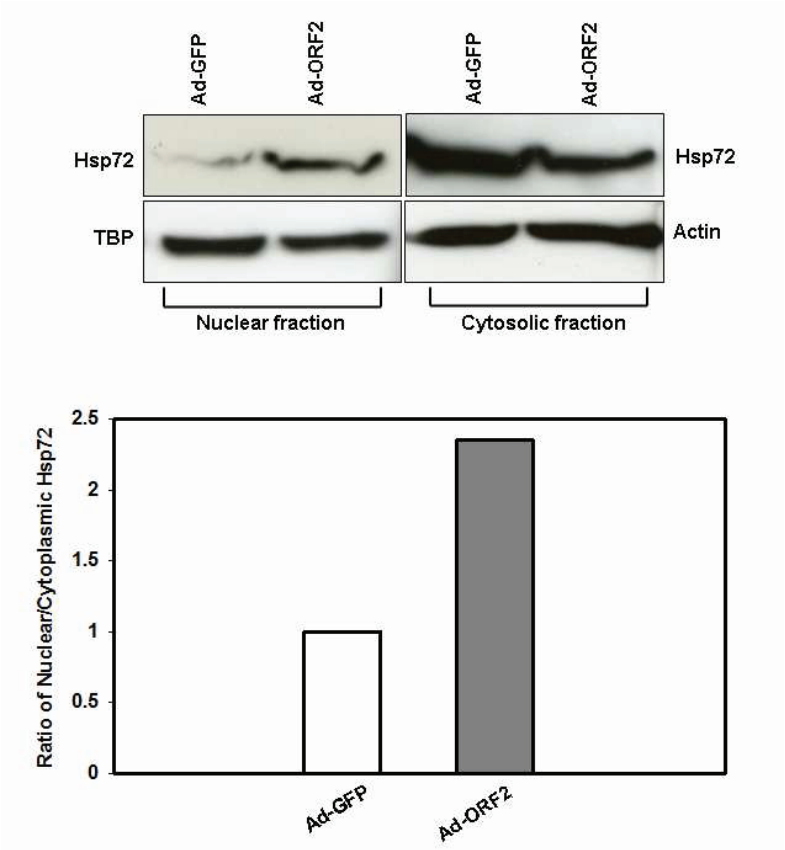

Figure 37: Hsp72 accumulates in the nuclear fraction of Huh7 cells transduced with Ad-ORF2. After 72 hpt, nuclear and cytosolic fractions were isolated and the nuclear accumulation of Hsp72 was detected by Western blot. Cytosolic actin and nuclear TBP were used as controls for equal loading. Signal intensities of Hsp72 for both nuclei and cytoplasm were quantified and normalized to the appropriate loading controls. The nuclear-cytoplasmic ratio was calculated as mentioned in methods.

5.3.8. Activation of the pro-apoptotic gene CHOP by ORF2 does not induce Bax translocation to mitochondria

Induction of CHOP activates the ER stress induced major apoptotic pathways. In addition overexpression of CHOP leads to decrease of anti-apoptotic B-cell CLL/lymphoma 2 (Bcl2) proteins level and induces the translocation of Bax to mitochondria. Under normal conditions Bax is located in the cytoplasm and under apoptotic events the Bax conformation changes to the pro-apoptotic state and is translocated into mitochondria. So we investigated whether HEV ORF2 induced CHOP activation may lead to any translocation of Bax in the Huh7 cell line. At 72 hours post transduction (hpt) of Huh7 cells either with Ad-ORF2 or an Ad-GFP, Bax

completely remained in the cytoplasm. In contrast, when the cells were treated with thapsigargin as a positive control, Bax was localized in the mitochondria (Fig. 38).

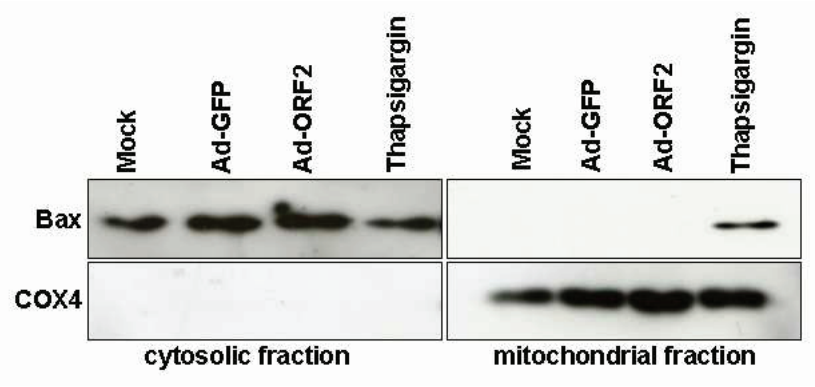

Figure 38: ORF2 does not induce the BAX translocation from cytosol to mitochondria. Huh7 cells were transduced with either Ad-GFP or Ad-ORF2 for 72 hours, and the uninfected cells were treated with thapsigargin for 48 hours prior to harvest. Mitochondrial and cytosolic fractions were isolated and the evaluation of Bax protein levels in cytosolic and mitochondrial fractions at 72 hpt. Thapsigargin served as positive control for ER stress induced Bax translocation to mitochondria. The mitochondrial protein COX4 was used as a control.

Moreover, Fig. 39 also shows that ORF2 did not induce apoptosis in all three cellines analyzed)

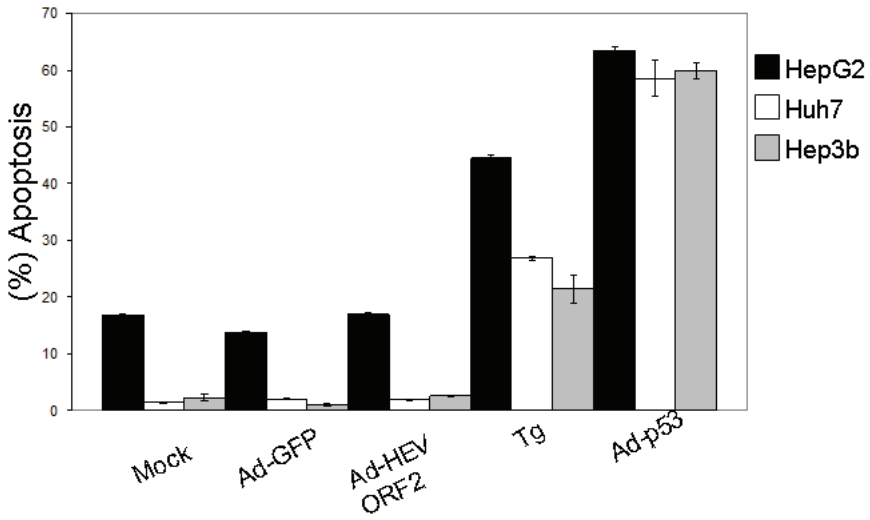

Figure 39: FACS analysis of hepatocyte derived cellines (HepG2, Huh7, Hep3b) upon ORF2 expression.

5.4. Discussion

The HEV ORF2 protein represents the capsid protein of the HEV. After translation ORF2 accumulates in the ER and a fraction of this protein is translocated back to the cytoplasm. For this purpose ORF2 has an ER translocation signal for these retro-translocation events (Surjit *et al.*, 2007). During the course of productive infection, the majority of viral proteins are synthesized by the host cell and processed through the cellular protein translocation machinery. Thus, ER is an essential organelle for the replication and maturation of this and many other viruses (Dimcheff *et al.*, 2004; von dem Bussche *et al.*, 2010). Proteins that are misfolded will be retained in the ER until they reach native conformation or are translocated back to the cytosol for proteasome mediated degradation proceses (Tsai *et al.*, 2002). HEV ORF2 protein was found to follow the retro-translocation pathway and remain in cytosol without being a substrate of the 26S proteasome (Surjit *et al.*, 2007). Overload with misfolded proteins in ER will cause ER stress and this will lead to the activation of UPR pathways. ORF2 protein is known to cause ER stress, and has been shown to activate ER chaperones like GRP78 and GRP94 (Surjit *et al.*, 2007). Up-regulation of these chaperones is known to be associated with the unfolded protein response which binds to and retains the misfolded protein in the ER. ER stress response in mammalian cells involves the major two phases of adaptation and apoptosis. During the adaptation stage cells will use the ER chaperones like GRP78 and GRP94 to refold the unfolded proteins to maintain the ER homeostasis (Little *et al.*, 1994; Ni & Lee, 2007). If, however this adaptation process fails, then the pro-apoptotic process will be initiated by the ATF6 and ATF4 dependent transcriptional activation of C/EBP homologous protein CHOP. My data show that ORF2 protein activated the full-length CHOP promoter and increased CHOP mRNA levels. In mammalian cells ER stress is induced by three different pathways that are mediated by PKR-like endoplasmic reticulum kinase PERK, activating transcription factor 6a and 6b (ATF6a and ATF6b) and IRE1a and IRE1b (Ma *et al.*, 2002; Yoshida *et al.*, 2001). Following ER stress IRE1 dimerizes and undergoes autophosphorylation following the activation of its endoribonuclease activity. Thus, the activated IRE1a and IRE1b will cleave the substrate precursor XBP-1 mRNA to mature XBP-1mRNA, and the spliced form of XBP-1 has potential transcription activity and can bind to the ERSE element of the CHOP promoter. Indeed, during ER stress activated PERK phosphorylates the subunit of eIF2α leading to a general attenuation of protein synthesis. This promotes translation of certain mRNAs such as mRNA encoding the transcription factor ATF4 which also binds to the AARE regulatory sites

of the CHOP promoter. Transcriptional regulation of the CHOP promoter is mainly triggered through the activation of the ERSE and AARE regulatory sites. We employed the promoter constructs with deletions of ERSE or transcription factor ATF4 binding region of CHOP and observed that deletion of the ATF4 region of CHOP reduced the full promoter activity. In addition, we also observed strong activation of AARE elements in ORF2 expressing cells. These results suggest that even though ORF2 protein induces the activation of ERSE element, the transcription factor ATF4 binding regions like AARE1 and AARE2 play a major role in the activation of CHOP promoter upon ORF2 expression. We analyzed the activation of the PERK pathway by ORF2 protein upon phosphorylation of eIF2α. We found that ORF2 protein increased the phosphorylation of eIF2α and thus confirmed the activation of PERK-eIF2α pathway. Since we have used the transient transfection system for our promoter activation assays, and to rule out an unspecific activation of CHOP due to the overexpression of a protein, we used the capsid protein of Chikungunya virus as a negative control which failed to activate the CHOP promoter. Our results clearly show the specific activation of the proapototic gene CHOP and its responsive elements. However, we did not find any indication of apoptosis in our cells expressing HEV ORF2. Activation of CHOP usually triggers the major apoptotic pathways and overexpression of CHOP will lead to down-regulation of Bcl2 protein levels and the translocation of Bax to mitochondria. The CHOP mediated death signals will be targeted to mitochondria, which may act as an integrator and amplifier of apoptotic pathways. However way mechanistic details for the direct involvement of CHOP in apoptosis are still unclear (McCullough *et al.*, 2001). Bax is mainly located in the cytoplasm under non-apoptotic conditions and translocates to mitochondria in response to the apoptotic stimuli. Thus, Bax is a major player of the ER stress mediated apoptosis. However, in our studies, we have not found the translocation of Bax to mitochondria due to the expression of ORF2 protein. This clearly suggests that activation of CHOP by ORF2 protein did not execute the apoptotic markers like translocation of Bax from cytosol to mitochondria. These findings are in agreement with recent findings of dengue virus infection. Dengue virus infection leads to activation of CHOP but fails to induce any apoptotic markers like suppression of Bcl2 protein levels, activation of caspases or cleavage of poly (ADP-ribose) polymerase (Pena & Harris, 2011). It has been reported that Hsp72 can inhibit the CHOP and TNF alpha induced apoptosis by binding to Bax and preventing its translocation to mitochondria (Gotoh *et al.*, 2004; Stankiewicz *et al.*, 2005). In agreement with this report we found that ORF2 expression up-regulated expression of Hsp72 and other chaperones. Expression levels of Hsp72 are rate limiting in control of ER stress and

its over expression helps cells to adapt to long term ER stress *in vivo* by enhancing the IRE1alpha/XBP1 branch of the UPR (Gupta *et al.*, 2011). Co IP experiments and *in silico* docking analysis revealed that ORF2 protein not only up-regulates, but also directly interacts with Hsp72. The molecular docking analyses have also shown that the C-terminal domain of the HEV ORF2 protein is probably responsible for the interaction with Hsp72 protein. Previous studies using molecular docking also showed that ORF2 protein interacts with chaperone GRP78 (Yu *et al.*, ; 2011). Thus the interaction of ORF2 with Hsp72 may be responsible to protect ORF2 expressing cells from apoptosis and to ensure correct protein conformation of newly synthesized ORF2 and other viral proteins. This is in agreement with previous reports that ORF2 protein is not a substrate of the 26S proteasome complex and that the protease sensitive or ubiquitination sites of HEV ORF2 were masked (Surjit *et al.*, 2007). Also this interaction may be linked with antigen presentation skills of Hsp72. Consistent with our microarray results and co-IP experiments, ORF2 protein also induced nuclear accumulation of Hsp72. Overexpresssion of Hsp72 results in cytoplasmic localization and under conditions of stress such as heat shock it will translocate to the nucleus. Exposure of toxicants like dimethylarisinic acid also causes the nuclear accumulation of Hsp72 and prevents apoptosis in human alveolar cells (Kato *et al.*, 1999). Nuclear accumulation of Hsp72 is mainly dependent on the phopshorylation status of tyrosine 524 and this nuclear translocation is important for the cell survival (Knowlton *et al.*, 2000). Taken together, HEV ORF2 protein can activate the pro-apoptotic gene CHOP through its stress responsive elements in an ATF4 dependent manner. Furthermore, HEV ORF2 induced activation of CHOP leads to the up regulation of chaperones like HS70B', Hsp72 and co-chaperone like Hsp40. In the light of our results we speculate that HEV ORF2 induces activation of Hsp72 and other chaperones may represent a survival mechanism in ORF2 expressing cells. Finally our studies will allow further investigation of the major heat shock protein like Hsp72 during HEV infection and could be exploited for therapeutic or diagnostic purposes.

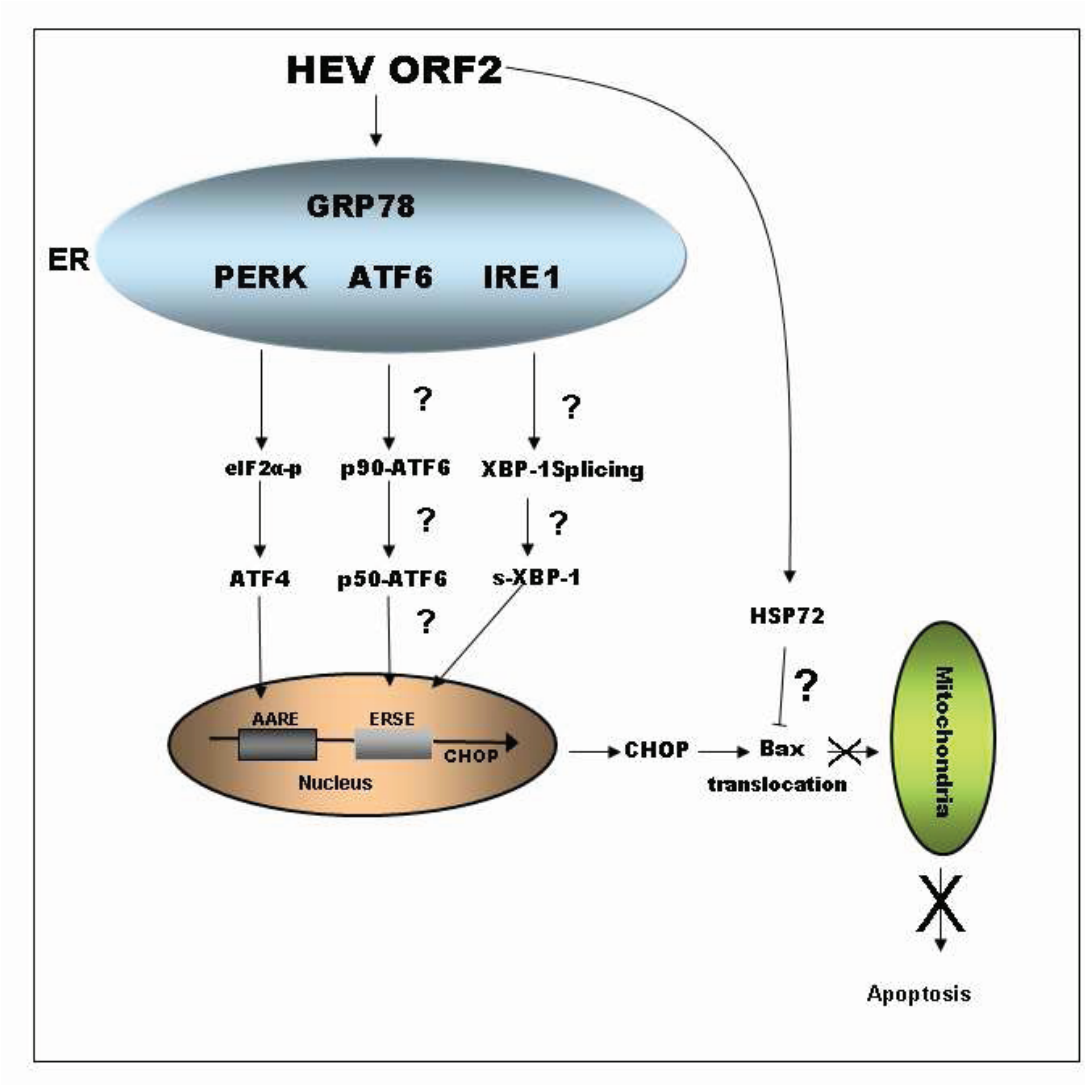

Figure 40: Model of HEV ORF2 protein induced activation of proapoptotic gene CHOP and Hsp72

6. Summary

All viruses require specific host factors for each phases of their life cycle. Identification of these host factors may not only unravel the mechanisms of viral infection but also may lead to the development of novel drug targets. I investigated the cellular requirements of two positive strand RNA viruses hepatitis C virus and hepatitis E virus. I have used the synchronized HCV infection system to study the transcriptional changes of host cells during the early stages of infection. Most of the genes were differentially expressed at 30 minutes post infection. These genes were functionally involved in the intracellular trafficking and endocytosis. Based on our microarray data, I have done siRNA screening of these identified host genes and found that knockdown of host gene KIFC1 could reduce the infectivity rate of HCV. Our data suggest that HCV requires the intracellular trafficking and endocytosis mechanisms for its successful entry into cells.

After entry, viruses require the host factors also for their replication. Replication of HCV occurs inside the membranous web. The non structural protein NS4B of HCV induces cellular rearrangements and this leads to the formation of membranous web. Since NS4B protein induced cellular effects are largely unknown, I have constructed the recombinant adenovirus which expressed the HCV NS4B protein, in order to screen the bona fide cellular targets. Microarray and phosphor-proteome array results showed an NS4B specific activation of Wnt/beta catenin signalling pathway, a well known oncogenic pathway that plays leading role in several cancers. These results suggest that NS4B regulates an oncogenic pathway.

In another line of investigation, I looked for the cellular effects caused by the capsid protein of the non-enveloped hepatitis E virus, ORF2 which is known to cause endoplasmic reticulum stress in cells. I found that ORF2 activates the pro-apoptotic CHOP promoter mainly though the AARE regulatory sites and that activation is dependent on the PERK pathway. To investigate the consequences of the pro-apoptotic effects, we performed a microarray analysis and the results showed an up-regulation of anti-apoptotic chaperones. In detail I found that ORF2 interacted with the chaperone Hsp72 and also induced its nuclear accumulation. These results clearly show that ORF2 induced activation of CHOP does not leads to apoptosis and this may be due to the counter action of anti-apoptotic chaperones. Finally these studies will allow further investigation of these identified cellular factors and could be exploited for therapeutic or diagnosticpurposes.

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9.Publications

1) **Lijo John**, Saijo Thomas, Ottmar Herchenröder, Brigitte M Pützer, Stephan Schaefer. Hepatitis E virus ORF2 protein activates the pro-apoptotic gene CHOP and anti-apoptotic heat shock proteins. Plos One September 2011.Volume 6.Issue 9.e25378

2) **Lijo John,** Simrat Dhillon, Aravind Patel, Stephan Schaefer. Global modulation of cellular transcriptional changes during synchronized HCV infection. (Manuscript under preparation).

10.Declaration

I hereby solemnly declare that this thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate, with due reference to the literature, and acknowledgement of collaborative research and discussions.

Rostock, 21.06.2011 **Lijo John**